

**MOUSE MODELS OF CHRONIC *TOXOPLASMA GONDII* (*T. GONDII*)
INFECTION: IMPLICATIONS FOR PSYCHIATRIC DISEASE**

By

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ABSTRACT

Toxoplasma gondii (*T. gondii*) is a parasitic protozoan that has been implicated in psychiatric disorders such as schizophrenia and bipolar disorder. While 33-50% of the world's human population is infected with the parasite, not everyone with serum antibody levels to *T. gondii* exhibit psychiatric symptoms. In order to better understand under what conditions infection with *T. gondii* may lead to symptoms of schizophrenia, we studied the effect of four different infection factors on mouse behavior. Factors studied include: i) *T. gondii* stains used in infection, ii) sex of the infected host, iii) age at which the host is infected, and iv) *T. gondii* cyst persistence or immune response to infection. We found that each of these factors can contribute to the development of different behavioral alterations in rodents.

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TABLE OF CONTENTS

1.	Introduction.....	1
2.	Mouse models of gene-environment interactions in schizophrenia (published in <i>Neurobiology of Disease</i>, 2013).....	6
2.1	Introduction	
2.2	Endophenotypes in animal models for GEI in schizophrenia	
2.3	Behavioral endophenotypes	
2.4	Electrophysiological endophenotypes	
2.5	Brain imaging endophenotypes	
2.6	Histological endophenotypes (GABA neuronal changes and spines)	
2.7	Current animal models of GEI relevant to schizophrenia	
2.7.1	Psychological stress	
2.7.2	Immune challenge	
2.7.3	Drug abuse	
2.8	Future directions	
2.8.1	Design weakness	
2.8.2	Technology development	
2.9	License Agreement	

3.	<i>Toxoplasma gondii</i> and cognitive deficits in schizophrenia: an animal model perspective (published in <i>Schizophrenia Bulletin</i>, 2012).	27
3.1	Introduction	
3.2	<i>T. gondii</i> infectious cycle	
3.3	<i>T. gondii</i> and cognitive deficits in humans	
3.4	Cognitive abnormalities in <i>T. gondii</i> -infected rodents	
3.4.1	Spatial learning and memory	
3.4.2	Olfaction based learning and memory	
3.4.3	Associative learning and memory	
3.5	<i>T. gondii</i> effects on the brain	
3.6	Future directions	
3.7	Conclusions	
3.8	License Agreement	
4.	<i>Toxoplasma gondii</i> strain-dependent effects on mouse behavior (published in <i>Folia Parasitologica</i>, 2010).	46
4.1	Introduction	
4.2	Materials and methods	
4.2.1	Animals	
4.2.2	<i>Toxoplasma gondii</i> culture and infection	
4.2.3	Infection determination	
4.2.4	Aversion to domestic cat urine	
4.2.5	Novelty-induced activity	

4.2.6	Spontaneous alteration and spatial recognition memory	
4.2.7	Anxiety	
4.2.8	Sensorimotor gating	
4.2.9	Statistical Analysis	
4.3	Results	
4.4	Discussion	
4.5	License Agreement	
5.	Sex-specific changes in gene expression and behavior induced by chronic <i>Toxoplasma</i> infection in mice (published in <i>Neuroscience</i>, 2012)	60
5.1	Introduction	
5.2	Experimental procedures	
5.2.1	Animals	
5.2.2	<i>Toxoplasma</i> culture and infection	
5.2.3	Sample preparation and microarray analysis	
5.2.4	Data mining	
5.2.5	Real-time PCR	
5.2.6	Behavioral assays	
5.2.6.1	Aversion to bobcat urine	
5.2.6.2	Sensorimotor gating	
5.2.6.3	Novelty-induced activity	
5.2.6.4	Olfactory acuity	
5.2.6.5	Social transmission of food preference (STFP)	

- 5.2.6.5.1 Jar and food preparation
 - 5.2.6.5.2 Habituation to food jars
 - 5.2.6.5.3 Feeding demonstrator mice
 - 5.2.6.5.4 Observing interaction between demonstrator and observer mice
 - 5.2.6.5.5 Food preference of observer mice
 - 5.2.7 Statistical analysis
- 5.3 Results
 - 5.3.1 Differential effects of *Toxoplasma* on gene transcription of male and female mice
 - 5.3.1.1 Gene expression profiling in frontal cortex in the initial cohort
 - 5.3.1.2 Canonical pathways among DEGs during *Toxoplasma* infection
 - 5.3.1.3 Identification of brain-related biological processes
 - 5.3.1.3.1 Nervous system development
 - 5.3.1.3.2 Neurological system process
 - 5.3.1.4 Real time PCR validation
 - 5.3.1.5 Comparison analysis between infected female and male mice
 - 5.3.2 Differential effects of *Toxoplasma* on the behavior of male and female mice

5.3.2.1	Effect of <i>Toxoplasma</i> infection on body weight and antibody titers of male and female mice	
5.3.2.2	Differential effects of <i>Toxoplasma</i> on the behavior of male and female mice	
5.4	Discussion	
5.4.1	Neurological effects in male mice	
5.4.2	Neurological effects in female mice	
5.5	Conclusion	
5.6	License Agreement	
6.	The Effect of Mouse Age during First Exposure to <i>Toxoplasma gondii</i> on Behavior and Brain Alterations.....	92
6.1	Introduction	
6.2	Material and methods	
6.2.1	Animals	
6.2.2	<i>Toxoplasma</i> tachyzoite purification	
6.2.3	Behavioral testing	
6.2.3.1	Novelty-induced activity of the open field	
6.2.3.2	Pre-pulse inhibition of the acoustic startle	
6.2.4	Brain processing for H&E staining	
6.2.5	Infectivity confirmation	
6.2.6	NR2 antibody measurements	
6.2.7	Monamine analysis	

6.2.8	Statistical analysis	
6.3	Results	
6.4	Discussion	
7.	One Minute Ultraviolet Exposure Inhibits <i>Toxoplasma gondii</i> Tachyzoite Replication and Cyst Conversion without Diminishing Host Immune Response (submitted to the <i>Journal of Experimental Parasitology</i>).....	107
7.1	Introduction	
7.2	Materials and methods	
7.2.1	Parasite purification	
7.2.2	UV-inactivation of parasites	
7.2.3	Trypan blue exclusion	
7.2.4	Parasite replication assay	
7.2.5	Parasite red/green invasion assay	
7.2.6	Animals	
7.2.7	Mouse inoculations	
7.2.8	Serology	
7.2.9	Parasite detection in mouse brain	
7.2.9.1	Cyst staining	
7.2.9.2	Real-time polymerase chain reaction	
7.2.9.3	Viable parasite recovery	
7.2.10	Statistical analysis	
7.3	Results	

7.4 Discussion

7.5 Conclusion

8. Immune Response to *Toxoplasma gondii* Infection Affects

Mouse Behavior.....127

8.1 Introduction

8.2 Materials and methods

8.2.1 *T. gondii* tachyzoite purification and UV-inactivation

8.2.2 Mouse inoculations

8.2.3 Behavior analysis

8.2.3.1 Novelty-induced activity of the open field

8.2.3.2 Sensorimotor gating of pre-pulse inhibition of the
acoustic startle

8.2.3.3 Dark-light box

8.2.3.4 Fear Conditioning

8.2.3.5 Passive Avoidance

8.2.4 Statistical Analysis

8.2.5 Infectivity confirmation

8.3 Results

8.4 Discussion

9. Concluding Remarks and Recommendations.....143

References.....	146
------------------------	------------

Curriculum Vitae.....	202
------------------------------	------------

TABLES AND FIGURES

Table 3.1	The effects of <i>T. gondii</i> infection on learning and memory in rodents.....	34
Fig 4.1	PRU- and ME49- infection increases mouse attraction to cat odor during the first 5 min of the 10-min trial.....	52
Fig 4.2	Differential effects of PRU- and ME49-infection on mouse behavior.....	53
Fig 4.3	No effect of infection on spatial recognition memory, anxiety, or sensorimotor gating.....	54
Table 4.1	Average body weight and <i>Toxoplasma gondii</i> antibody titres of infected and control mice.....	55
Table 5.1	Number of differentially expressed RefSeq genes and altered canonical pathways.....	72
Fig 5.1	Enriched biological processes and their relationships involved in nervous system development by <i>Toxoplasma</i> infection.....	75

Fig 5.2	Enriched biological processes and their relationships involved in neurological system process by <i>Toxoplasma</i> infection.....	77
Fig 5.3	Correlation between microarray and real-time PCR data.....	78
Table 5.2	Average body weights (g) of infected and control mice throughout the course of behavioral testing.....	80
Fig 5.4	Effect of infection on aversion to cat odors during a 60-min trial.....	81
Fig 5.5	Effect of infection on social transmission of food preference (STFP).....	82
Fig 5.6	Female infection increased locomotor activity in the open-field arena compared with male.....	83
Fig 5.7	Infection did not influence prepulse inhibition in A) females and B) males.....	84
Fig 6.1	Age of host when infected with <i>T. gondii</i> affects pre-pulse inhibition (PPI) susceptibility to MK-801 and amphetamine.....	99

Table 6.1	Mouse weights, anti-IgG levels in serum, and cyst numbers.....	100
Table 6.2	Monamine changes in the striatum and hippocampus of mice infected as juveniles and adults	102
Fig 6.2	Anti-NR2 antibody in serum.....	103
Fig 7.1	Inactivation apparatus.....	110
Fig 7.2	TrypanBlue exclusion of UV inactivated tachyzoites.....	116
Fig 7.3	UV exposure prevents <i>in vitro</i> tachyzoite replication.....	117
Fig 7.4	Anti-Toxoplasma IgG levels at 20 days post infection (dpi).....	119
Fig 7.5	Kinetics of anti-toxoplasma IgG levels.....	120
Fig 7.6	Kinetics of mouse weight.....	121
Fig 7.7	Parasite cyst presence and parasite viability from mouse brain.....	122
Fig 7.8	Real-time PCR of Toxoplasma 5S rRNA gene.....	123

Fig 8.1	<i>T. gondii</i> infection increases mouse anxiety in the dark-light box.....	135
Fig 8.2	Associative learning and memory impairment in <i>T. gondii</i> infected mice.....	136
Fig 8.3	Contextual and cued fear conditioning is not affected by <i>T. gondii</i> infection.....	137
Fig 8.4	MK-801 disruption in pre-pulse inhibition of the acoustic startle.....	139
Table 8.1	Mouse weight and anti- <i>Toxoplasma</i> IgG serum levels.....	140

CHAPTER 1:

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite found world-wide. *T. gondii* can infect any warm-blooded mammal, humans included. Approximately 33-50% of the world's human population is infected, as determined by serum antibody levels to the parasite. While already a large percent of the population is infected, it is postulated that due to climate change we may see an increase in the number of infected individuals (1).

Infection with *T. gondii* has long been considered a major health concern for only immune-compromised and pregnant individuals. *T. gondii* can harbor in the brain of immune-competent individuals as semi-dormant parasite cysts. For those with compromised immune systems, the *T. gondii* cysts can rupture and stage convert into the actively replicating tachyzoite. The tachyzoites can then lead to *Toxoplasma* encephalitis (2). For pregnant individuals, *T. gondii* infection can adversely affect the fetus. For instance, *T. gondii* infection during the first trimester can lead to mental retardation, seizures, blindness, and death later in the child's life (3).

It is now realized that *T. gondii* may also affect individuals with healthy immune systems. The parasite has been associated with various alterations in human behavior, and has been implicated in psychiatric disease, such as schizophrenia and bipolar disorder (4, 5). Evidence associating *T. gondii* infection with psychiatric disorders includes *in vitro*, *in*

vivo, clinical, and epidemiological studies. The greatest indication lies in studies looking at antibody levels to *T. gondii* in patients with schizophrenia and healthy age matched controls. Combining results from independent studies around the world revealed an increased risk [odds ratio (OR) = 2.73] of having schizophrenia if antibodies to *T. gondii* are present in serum (6). In addition, it has been observed that some people with acute toxoplasmosis also exhibit delusions and hallucinations reminiscent of schizophrenia (4). It has also been shown that anti-psychotics and mood stabilizers used to treat patients with psychiatric disorders have anti-*Toxoplasma* activity *in vitro* and *in vivo*. For example, *in vitro* replication of the parasite has been shown to be inhibited by anti-psychotic (haloperidol) and mood stabilizers (valproic acid) (7). Also, these antipsychotics and mood stabilizers acted similarly to anti-*T. gondii* drugs in reversing *T. gondii*-induced behavioral changes in rats(8). While these data suggest a role of *T. gondii* infection in psychiatric disorders, one cannot ignore the fact that not everyone infected with *T. gondii* develops a disorder. It is therefore plausible that only under certain circumstances will *T. gondii* infection lead to the development of symptoms of psychiatric disorders.

The ability to tease apart cause and effect (e.g. *T. gondii* infection and psychiatric symptoms) in humans is virtually impossible. For this reason, rodent models are important and useful tools as they can be manipulated in a controlled setting. In addition, when dealing with *T. gondii* infection, rodents are a suitable model because the parasite will undergo the same stage conversions as in humans. Mouse models of schizophrenia and rodent models of cognitive deficits due to *T. gondii* infection are discussed in

chapters 2 and 3. Using mice as a model, we sought to study the contribution of different factors to behavioral alterations in rodents.

The first factor we looked at was the *T. gondii* strain (Chapter 4). There are many different strains of *T. gondii* in the world that are grouped into clades based on genetic similarity. Parasites between clades differ genetically by less than 1 percent. However, they are different in virulence and, relatedly, cytokine induction of the host (9, 10).

Although strains from all clonal lineages have been isolated from humans, the majority of human toxoplasmosis cases have been associated with strains from the Type II clade (11). It is conceivable that although all *T. gondii* strains are capable of infecting humans, specific *T. gondii* strains may lead to the development of particular clinical presentations of psychiatric disorders in humans.

The second factor we looked at was the sex of the host (Chapter 5). Studies looking at behavioral changes in men and women with serum antibodies specific to *T. gondii* have found a number of changes (5, 12-16). For instance, it was found that for both men and women, infection with *T. gondii* can increase the risk of being in a traffic accident (17). Surprisingly, infected men showed increased depression and were more withdrawn as compared to uninfected men, a finding not present in women. Also, while infected men showed less self-control and were less tidy, infected women had more self-control and were more tidy than their uninfected counterparts (18). Based on the clinical data, it is apparent that infection with *T. gondii* affects men and women differently.

The third factor we looked at was the age the host is first exposed to *T. gondii* (Chapter 6). Onset of schizophrenia is during young adulthood, with onset for men in the late teens and for women in the early twenties. At either of these ages, the brain is developmentally mature. In contrast, during adolescence the brain is immature and changing. Through this period, changes include maturation of interneurons and mesocortical dopaminergic projections and pruning of glutamatergic synapses (19). It is conceivable that infection when the brain is mature may have little impact, but pathogen insult during the adolescent period could have consequences on brain development that lead to the onset of psychiatric disorders during young adulthood.

The fourth factor we looked at was the contribution of the immune response to *T. gondii* infection versus the persistence of parasite cysts in the brain (Chapter 7 and 8). *T. gondii* cysts have been shown to express tyrosine hydroxylase homologs and contain dopamine (20). As an imbalance in dopamine is associated with psychiatric disorders, it is thought the parasite manipulates behavior through production of excess dopamine. However, *T. gondii* cysts have yet to be found in the brains of individuals with psychiatric disorders. To date, a serological response to specific *T. gondii* cyst antigens is used to identify those with (or whom might have had) parasite cysts (21). Recently it has been found that mice infected with an attenuated strain of *T. gondii* that renders them incapable of producing parasite cysts exhibit similarly altered behavior to mice that have parasite cysts (22). It is therefore plausible that while many humans are seropositive to *T. gondii*, perhaps only those unable to clear the parasite cysts from the brain are the ones who develop psychiatric disorders.

Using mice as a model host, we sought to study the contribution of 4 different factors to behavioral alterations in rodents. We believe that first understanding what condition(s) would lead to specific behavior changes will aid in elucidating how *T. gondii* infection alters behavior and in creating and testing pre-clinical compounds to be used in patients to ameliorate symptoms of psychiatric disorders.

CHAPTER 2:

Mouse models of gene-environment interactions in schizophrenia

(published in Neurobiology of Disease, 2013)

Abstract

Gene-environment interactions (GEI) likely play significant roles in the pathogenesis of schizophrenia and underlie differences in pathological, behavioral, and clinical presentations of the disease. Findings from epidemiology and psychiatric genetics have assisted in the generation of animal models of GEI relevant to schizophrenia. These models may provide a foundation for elucidating the molecular, cellular, and circuitry mechanisms that mediate GEI in schizophrenia. Here we critically review current mouse models of GEI related to schizophrenia, describe directions for their improvement, and propose endophenotypes provide a more tangible basis for molecular studies of pathways of GEI and facilitate the identification of novel therapeutic targets.

2.1 Introduction

Genetic and environmental factors, as well as their interplay, contribute to individual differences in vulnerability to psychiatric disease (23, 24). Gene-environment interplay is a term that encompasses several models (25, 26). These include altering gene expression by environmental factors via epigenetic mechanisms, additive interaction between genetic and environmental factors, gene-environment correlations or genetic control of exposure to the environment, and genetic control of sensitivity to the environment (25-27). Genetic moderation of individual susceptibility to the adverse or protective effects of the environment provides an explanation for most examples of what have been termed genotype-environment interactions (GEI) (26). This chapter will focus on mouse models that mimic GEI etiologically relevant to schizophrenia.

GEI are difficult to assess in clinical studies (28, 29). Animal models offer a means of elucidating the contribution of genes, environmental factors, and their interactions on pathogenesis of psychiatric disease (30, 31). As technology has and continues to develop, a number of genetic models can be made to use in conjunction with environmental insults to look at GEI. However, the most useful models to study human disease should incorporate genetic changes and environmental components that are etiologically relevant (32, 33).

As schizophrenia and related disorders are increasingly considered as disorders that include etiologies associated with brain development, rodent models with manipulation in

genes involved in neurodevelopment may be useful (19, 34). In a similar vein, it is important to take developmental considerations into account when interpreting environmental effects that can be variable in different age groups. For schizophrenia, pre- and postnatal events that induce psychological stress seem to exacerbate symptoms in adulthood, infectious etiologies have predominantly been associated with prenatal exposure, and illicit use of drugs has been found to be relevant during early adolescence (27, 35).

We have also proposed that promising animal models of GEI would include etiologically relevant genetic and environmental risk factors that would have strong functional impact and converge on common signaling pathways (33). Thus, we will overview mouse models that combine genetic variations with psychological stressors (36-38), immune activation (39, 40) and cannabis exposure (41-43). The present chapter will critically evaluate the weakness of the current approaches and will suggest possible new directions in the development of GEI models with a particular focus on endophenotypic measures that are thought to be instrumental for mechanistic studies, more readily translatable to human conditions, and targetable by therapeutics (44).

2.2 Endophenotypes in animal models for GEI in schizophrenia

As it is impossible to faithfully create the key features of schizophrenia such as hallucinations and delusions in animals, a more tractable and promising approach that has been gaining attention is to model brain circuitry, and cellular and molecular alterations

associated with the disease. Such alterations can be broadly termed as endophenotypes (45). In the context of GEI animal models, the main advantage of endophenotypes is that such abnormalities can be objectively measured in patients and faithfully replicated in animals to help decipher the underlying mechanisms of GEI. Here, we briefly overview several endophenotypes that are relevant to schizophrenia and may be utilized in basic studies of GEI.

2.3 Behavioral endophenotypes

Despite the obvious reservations about reproducing human emotion and cognition in animals, some behaviors are conserved in humans, primates, and rodents. Changes in some evolutionarily preserved behaviors are observed in patients and can be experimentally induced in animals, including hyperactivity, impaired pre-pulse inhibition (PPI) of the acoustic startle response, deficient social interaction, and cognitive deficits (23). Although these behavioral alterations are not specific to schizophrenia, their objectivity and reproducibility make them useful endophenotypes. For example, as PPI is diminished in patients with schizophrenia (46), testing for PPI impairment remains a critical component of any animal study of schizophrenia (47-49). Similarly, given that cognitive deficits are debilitating and the least treatable abnormalities in schizophrenia (50, 51), there is a growing appreciation for developing more sophisticated tests to evaluate cognitive processes, including working memory and attention (52, 53).

Behavioral endophenotypes have been widely used in animal models of major psychiatric diseases and animal models of GEI. Still, more work is needed to develop translatable

and reproducible behavioral endophenotypes for negative symptoms and cognitive deficits of the disease (54).

2.4 Electrophysiological endophenotypes

Patients with schizophrenia display deficits in processing external stimuli from the environment (55-57). These deficits can be assessed with auditory event-related potentials (ERPs) methodology. Reductions in N100 or mismatch negativity and changes in theta and gamma frequency have been proposed as electrophysiological endophenotypes relevant to schizophrenia (58-60). Such endophenotypes can now be successfully measured in animals (45, 61, 62). Abnormal functional inter-regional connectivity has also been implicated in the pathophysiology of schizophrenia (63, 64). Newly developed tools enable us to study functional connectivity in laboratory animals. For example, reduced synchronization of neural activity between the hippocampus and the prefrontal cortex during a working memory task was found in a mouse model of the 22q11.2 deletion (65). Another electrophysiological method that is being developed to examine the pathophysiology of cognitive impairment is stimulus specific response potentiation. This tool can be used to assess long-lasting, experience dependent plasticity in the primary visual cortex of rodents (66). These techniques are only beginning to be utilized in animal models of psychiatric disease but hold the significant promise of objectively evaluating effects of GEI at the circuitry level particularly in combination with *in vivo* imaging.

2.5 Brain imaging endophenotypes

The introduction of neuroimaging has revolutionized brain research, providing significant insights into the pathophysiology of psychiatric diseases (67-70). Adaptation of neuroimaging to rodents has enabled researches to observe *in vivo* longitudinal changes at the organ, cell, and molecular levels (67, 71). Magnetic resonance imaging (MRI) has been used to assess volumetric changes in the lateral ventricles and brain regions in several animal models for schizophrenia (72-75). The animal variant of positron emission tomography (PET), micro-PET, has been helpful in assessing neurochemical changes (e.g. receptor binding) that resemble PET findings in patients (76). The simultaneous use of MRI and micro-PET in rodent models of schizophrenia may provide valuable information on changes in receptor density and neurotransmitter and metabolite concentration due to specific genetic or environmental manipulations (67). The significant advantages of *in vivo* neuroimaging are longitudinal monitoring of the brain alterations of GEI and the treatment effects in the same animal. However, the cost of neuroimaging is high and likely deters wider use of this technology. Also, low resolution of the images may make subtle changes difficult to assess, requiring the use of traditional histological methods.

2.6 Histological endophenotypes (GABA neuronal changes and spines)

Histological analysis provides insight into specific cell modifications (e.g. number or morphology) that still are unavailable with *in vivo* imaging. Decreased immunoreactivity of parvalbumin positive gamma-aminobutyric acid (GABA) interneurons in the cortex and hippocampus are commonly observed in postmortem brains of patients with

schizophrenia (77). This histological hallmark of the disease has been reported for many animal models of schizophrenia and is a promising endophenotype for GEI research. Similarly, abnormal maturation, morphology, and functions of dendritic spines and synapses have been associated with major psychiatric diseases (78). Although specific dendritic or synaptic abnormalities for schizophrenia have not been found, understanding the molecular underpinnings of synaptic pathology has been suggested to be a promising direction for future research on identifying new therapeutic targets (79, 80).

2.7 Current animal models of GEI relevant to schizophrenia

A number of animal models of schizophrenia have focused on manipulating genes in order to determine their contributions to disease. However, it is becoming increasingly apparent that genetic manipulations alone do not faithfully reproduce many endophenotypes of schizophrenia. Combining genetic risk factor with environmental adversities such as psychological stress, immune challenge, or drug exposure, provides a better approach to modeling the complexity and heterogeneity of schizophrenia. Here we review the GEI models grouped according to the type of the environmental factor used.

2.7.1 Psychological stress

Pre- and post-natal psychological stress has been implicated in the etiology of schizophrenia (38, 81). To better understand the role of stressful experience on the development of schizophrenia, a number of groups have exposed genetically modified mice to various types of stressful treatment either *in utero* or postnatal.

One group sought to determine whether a combination of prenatal variable stress and a point mutation in the synaptosomal-associated protein of 25 kDa (SNAP25) would lead to behavioral endophenotypes reminiscent of schizophrenia. They found that prenatal stress and the genetic mutation acted synergistically to produce deficits in sociability and social novelty that were not seen in unchallenged mutants or stressed control animals. In addition, prenatal stress and the point mutation additively increased PPI impairment already present in mutant mice (82). This study was one of the first to demonstrate synergistic and additive effects of genetic and environmental factors on behavioral endophenotypes relevant to schizophrenia. However, the model has the limitations for molecular mechanistic studies of interactions between the point mutation in the *Snap 25* gene in the mouse embryo and an array of signaling pathways activated by stress in pregnant dams as the point mutation may or may not have functional effects on the intracellular stress response signaling. In addition, it is important to note that while this GEI model revealed impaired PPI, the point mutation in the *Snap 25* gene has not been associated with schizophrenia. Rather, a reduction in SNAP25 expression has been found in the postmortem samples (83).

The interaction of postnatal stress and deletion of a schizophrenia risk gene, *Neuregulin-1* (NRG1) has been looked at by a few groups. Desbonnet and associates (2012) examined the behavioral effects of interactions between repeated social defeat during adolescence (postnatal day 35-45) and deletion of NRG1. Independent of adolescent stress, mice deficient in NRG1 showed increased activity levels, deficits in PPI, and a reduction in social novelty preference. Interestingly, adolescent stress alone did not have an impact on

these behaviors. Rather, a combination of adolescent stress and NRG1 deletion was needed to observe decreased activity, poorer spatial working memory, and diminished sucrose preference (84). In NRG1 mice, stress also influenced spleen cytokine response to concanavalin A and altered expression of mRNAs encoding for brain cytokines and brain derived neurotrophic factor. In contrast, acute restraint stress during adulthood (3-4 months and 6-7 months of age) led to different effects. Older NRG1 mutants were less susceptible to the effects of stress on anxiety-related behaviors than younger mutants (85). These studies highlight that varying the time of exposure to stressful events leads to differential behavioral alterations in the same transgenic mouse model. A caveat of the NRG1 deficient model is lack of tissue specificity. NRG1 has an important role in cardiac development (86), with homozygous deletion of NRG1 leading to embryonic death from cardiac problems in mice (87). It is conceivable that NRG1 heterozygous mice have heart problems that affect brain functions. Future studies with this model should try to delineate the specific mechanisms of GEI (e.g. a role of NRG1 in the stress signaling pathways).

Our group has recently provided a mechanistic insight into how a mild isolation stress during adolescence affects the mesocortical projection of dopaminergic neurons in which DNA hypermethylation of the tyrosine hydroxylase gene is elicited only if stress is combined with expression of a promising genetic risk factor, mutant *Disrupted-In-Schizophrenia-1* (DISC1) under the prion promoter. These molecular changes could contribute to several neurochemical and behavioral deficits that are blocked by a

glucocorticoid receptor antagonist. We propose that the biology and phenotypes of the mouse model resemble those of psychotic depression (88).

2.7.2 Immune Challenge

Viral infections have been implicated in the pathogenesis of schizophrenia (4, 89, 90).

One possible mechanism whereby viral infections can contribute to schizophrenia is via secretion of pro-inflammatory factors by the host in response to infection (39). Notably, elevated levels of maternal serum cytokines have been associated with children who later develop schizophrenia (39, 91, 92). Mouse models of immune activation due to viral infection use the viral mimic polyinosinic:polycytidylic acid (poly I:C). Poly I:C is a synthetic analog of double-stranded RNA and, similar to a live virus, interacts with toll-like receptor (TLR) 3 expressed by cells of the immune system (93).

Prenatal immune activation with poly (I:C) has been used in a few models of GEI. One study combined prenatal administration of poly (I:C) during early gestation stage (embryonic day 9, E9) and expression of mutant human *Disrupted in Schizophrenia 1* (DISC1) and evaluated behavioral, brain imaging, spine density, and molecular markers that could be relevant to psychiatric disease. The initial hypothesis of the authors was that a combination of prenatal immune activation and mutant DISC1 would synergistically produce stronger phenotypes consistent with aspects of schizophrenia. However, prenatally exposed mutant DISC1 mice showed a set of neurobehavioral alterations that were not consistent with schizophrenia and were more similar to affective disorders, including increased anxiety and depression-like behaviors, decreased social interaction,

decreased volumes of amygdala and periaqueductal gray, and altered responses to acute restraint stress (94). Taking advantage of the inducible system in this transgenic mouse model, the authors also demonstrate that life-long expression of mutant DISC1 is required to produce the affective behaviors in mice. An unexpected outcome of the study was an emergence of previously unseen behavioral and brain abnormalities in mutant DISC1 mice. This result illustrates an important consideration for evaluating GEI models. Combining genetic and environmental factors can lead to the development of new phenotypes rather than exacerbation of pre-existing abnormalities. A potential caveat of the study is the predicted timing of expression of mutant DISC1 in selective neuronal population. Although the authors show expression of mutant DISC1 as early as E9 when poly (I:C) treatment is applied, additional studies are needed to better understand the regional and time-related expression pattern of this mutant protein during early gestation to inform future molecular studies with this model. A more detailed evaluation of timing of GEI in this model would be important as well, including the question if turning off expression in adult mice might improve some brain and behavior alterations.

Meyer's group also examined interactions between prenatal immune activation during late gestation stage (E17) and the heterozygous deletion of nuclear receptor related 1 (*Nurr1*), gene that encodes for a transcription factor involved in dopaminergic neuronal development through activation of tyrosine hydroxylase (95). The combination of the genetic and environmental factors was found to not only exert additive effects on increased locomotor activity and deficits in sensorimotor gating, but also to produce synergistic effects on attentional shifting and sustained attention. In addition, *Nurr1*

deficiency in conjunction with prenatal poly (I:C) administration lead to improper development of prefrontal cortical and ventral striatal dopamine systems. This was suggested via reduced dopamine receptor 2 (D2R) in the nucleus accumbens and reduced tyrosine hydroxylase and increased catechol-O-methyltransferase (COMT) in the medial prefrontal cortex (95). These molecular deficits are consistent with findings in patients with schizophrenia (96). However, a caveat of this model is the limited human relevance of the deletion as only missense mutations in the *NURR1* gene have been associated with schizophrenia and manic-depressive disorder (97). Still, given a leading role of DA dysregulation in the pathophysiology of schizophrenia, one can foresee future research with this model of GEI in identifying the molecular mechanisms whereby NURR1 and the innate immune response from poly (I:C) administration work together to modulate maturation of DA neurons.

Postnatal immune activation has also been studied in conjunction with DISC1. One study looked at neonatal (postnatal days 2-6) treatment with poly (I:C) in transgenic mice constitutively expressing mutant DISC1 (98). Focusing on behavior and histological measures, they found that DISC1-poly (I:C) exposed mice showed reduction in working memory, increased susceptibility to MK-801 induced hyperactivity, and a decrease in parvalbumin positive GABA neurons in the medial prefrontal cortex. These deficits were not observed in mutant DISC1 mice without immune stimulation or control immune stimulated mice, suggesting synergistic effects. The authors focused on the characterization of schizophrenia-like behaviors in poly I:C/DISC1 mice and did not provide a detailed description of possible neurobehavioral effects following the single

factor exposure. In a follow-up study, cognitive impairment and MK-801 induced activity observed in poly I:C/DISC1 mice were improved by administration of clozapine (99). The ability of currently approved antipsychotic medication to ameliorate behavioral endophenotypes suggests that this GEI mouse model may be useful in the development of new therapeutics. A limitation of this model is that there is insufficient epidemiological support for childhood viral infection to increase the risk for schizophrenia. From a methodological standpoint, the model needs to control for possible maternal effects. For example, if poly (I: C) administration of pups resulted in sickness behavior, nursing dams may have treated sick and healthy pups differently, which may confound the outcomes of the study.

Use of poly (I:C) administration in mouse models has distinct advantages in reproducibility, reliability, and simplicity. However, there are disadvantages. The route of administration (e.g. intraperitoneal or intravenous) does not mimic the route by which pregnant women are most likely to get infected by a virus (e.g. respiratory). The elicited immune response may differ from that produced by a live viral infection (100). To overcome the limited immune response due to poly (I:C), some have used live microorganisms to model human infections in animals (101, 102). A potential issue of this approach is our limited options for using live pathogens in rodents. Many relevant infections are species-specific and thus do not involve the same mechanisms of invasion, replication and dissemination (e.g., herpes simplex virus 1). In addition, C57BL/6 mice that are predominantly used in genetic models easily succumb to human pathogens (e.g., influenza virus or *Toxoplasma gondii*), complicating longitudinal behavioral studies. A

possible solution to this issue is to transfer the mutation in question to less susceptible Balb/C mice by a series of backcrosses or to treat animals with attenuated pathogens to better mimic the specificity and magnitude of immune activation.

2.7.3 Drug abuse

Cannabis use may precipitate schizophrenia in genetically susceptible individuals (43). In order to mimic this interaction, animal models utilize Δ -9-tetrahydrocannabinol (Δ^9 -THC) that is the principal psychoactive component of the cannabis plant (103). A functional variant in the catechol-O-methyltransferase (*COMT*) gene (i.e., a switch in the amino acid methionine to valine in position 158) leads to greater COMT activity in the prefrontal cortex and resultant dysfunctions in humans (104). It has been found that adolescent cannabis exposure in individuals with the genetic variant in the *COMT* gene synergistically increased risk of psychosis (41).

The interactions between COMT and cannabis exposure were evaluated in a series of elegant studies by John Waddington and colleagues. They found that COMT knock out (KO) mice chronically exposed to THC during adolescence exhibited behavioral and histological alterations reminiscent of schizophrenia (54, 105). COMT KO mice exposed to THC as adolescents showed decreased spatial working memory and anxiety, which were not seen in mice treated with THC in adulthood (54). Adolescent exposure to THC in COMT KO mice led to decreased size of dopaminergic neurons in the ventral tegmental area and parvalbumin positive GABA neurons in the prefrontal cortex, consistent with the similar endophenotypes in patients with schizophrenia (105). These

results further indicate the importance of timing of GEI and the utility of endophenotypic measures. As with many GEI models described, a caveat of this mouse preparation is a deletion of the *COMT* gene rather than a knock-in expression of the Val158Met polymorphism seen in humans (106).

A number of studies were performed to assess the effects of acute and chronic THC treatments in adolescent and adult heterozygous *Nrg1* KO mice, with a focus on behavior endophenotypes (107-111). A greater decrease in locomotion, elevated anxiety in the light-dark box test, and enhancement of PPI were more pronounced after acute THC treatment in adult *NRG1* male mice compared to wild type littermates. A follow-up study did not find significant effects of acute THC in female mutant mice, indicating a major role of sex in modulating neurobehavioral outcomes of GEI in many mouse models. The same group of investigators also evaluated the behaviors in *NRG1* mice chronically treated during adolescence with THC and found few effects (111). When adult mice were given chronic injections of a cannabis constituent cannabidiol (CBD) that has anxiolytic and antipsychotic properties (110), it did not alter the pre-existing behavioral abnormalities but selectively enhanced social interaction in *NRG1* heterozygous (HET) mice. In addition to behavior, this group looked at receptor profiling versus autoradiography. They found that chronic THC exposure during adolescence increases binding in *NRG1* KOs and decreases binding in wild-type of serotonin receptors in the cortex (110). The study also highlights a recurrent issue with GEI models, namely, that GEI do not always lead to exacerbation of pre-existing conditions but can be protective or produce new changes that were not originally present in mutants. A potential weakness of

this model is lack of human studies on association between cannabis and genetic variants in the *NRG1* gene as well as our poor understanding of molecular interrelatedness between this gene and CB receptors signaling. Still, this type of interplay could provide interesting information on GEI relevant to schizophrenia (112).

2.8 Future directions

Recent progress in psychiatric genetics and epidemiology has facilitated the development of animal models of GEI relevant to schizophrenia. Although these models have provided some important insights, many caveats of recent preparations need to be addressed in the future studies. We would like to group the current issues into the methodological and technological ones. The former group calls for improvement of design and analysis of the existing models, while the latter one requires new technological approaches.

2.8.1 Design weaknesses

The field of animal models of GEI is still in its infancy but is rapidly expanding. As it continues to expand, it can learn from the studies of genetic models of psychiatric disease to avoid the known pitfalls. These include varying mouse background strains, history of breeding (e.g. inbred, outbred), and housing conditions. We believe that utilization of standard endophenotypic measures may not only help minimize variability in effects of GEI but also bring in new model organisms to study the molecular mechanisms of GEI across species (e.g., worms, fruit flies, zebrafish).

Another issue is the under appreciation of sex-dependent effects. Many models demonstrate sex-dependent alterations, yet most GEI models focus on a single sex. Future research in GEI models should address if sex-specific abnormalities result from the effects of GEI or their individual components on actions of gonadal hormones or from the modulatory influence of sex hormones on the pathways involved in GEI. Advancing our knowledge of the underpinnings of sex differences in psychiatric disorders could help uncover risk/protective factors and develop better treatments.

A promising direction in optimizing GEI model is to develop behavioral tests that are based on the natural murine behavioral repertoire (84). It has been proposed that more accurate behavioral evaluation of animal models of neuropsychiatric disorders should include species-specific behavioral approaches (113). Rodent specific tests will complement clinically relevant paradigms by providing more sensitive tests for analysis of underlying pathology (84)

Further, schizophrenia is increasingly considered a disorder of brain development, animal models with manipulation in genes involved in neurodevelopment are going to be most informative (19, 34). It is important to take developmental considerations into account when interpreting environmental effects that vary across different time points (27, 35). In the past, addressing time-dependent interaction in GEI models has been achieved by changing the time when genetically modified animals are challenged with an environmental adversity. Future studies should also attempt to regulate timing of the

effects of a specific mutation as exemplified by a recent study with inducible expression of mutant DISC1 in mice prenatally exposed to maternal immune activation (94).

Combining an environmental challenge with a genetic mutation can produce both protective and adverse effects. Instead of predicted synergistic effects, a GEI design can, for example, result in phenotypes previously unseen in unchallenged mutant mice (54, 94, 105). Such results should be anticipated by designing future GEI experiments to avoid a trap of a limited set of pre-planned tests used to “capture” specific disease-related phenotypes. Appearance of new brain and behavioral phenotypes, particularly while using the genetic mutation implicated in various psychiatric conditions, could inform us about the role of environment in bringing about diverse clinical outcomes in patients with the same mutation. The Scottish pedigree with the disruption of DISC1 due to the chromosomal defect is a most prominent example of such a possibility (114).

The focus of most published GEI research has been on risk factors. However, the contribution of protective factors is also important and has so far been relatively neglected, although there are some exceptions. Identification of genes conferring resilience to schizophrenia-related abnormalities is a new emerging research to uncover unrecognized molecular targets (115). New models using neurodevelopmental factors of resilience are clearly needed to advance this promising research. In this context, the role for environment enrichment in ameliorating/rescuing genetically produced abnormalities has been recently reviewed (116). Combining this type of preventive “therapy” with current GEI models would be interesting in determining whether environmental

enrichment can overcome effects caused by aversive environmental insults (e.g. psychosocial stress, infection, drug use) and offer a novel approach to treatments of the cognitive and negative symptoms that are resistant to the current antipsychotics (80, 117).

2.8.2 Technology development

The methodologies to manipulate the mouse genome at different levels of its organization (DNA, RNA regulatory sequences) are constantly improving. Simple knockout and transgenic technologies while remaining the workhorse of mouse genetics produce artificial systems inconsistent with the molecular pathology of schizophrenia. New models with mutations in regulatory elements in candidate genes with more subtle and temporally specific expression changes or human genetic variants knock-in models will better reflect the complex genetic and molecular mechanisms of schizophrenia (106). Therefore, time-dependent, circuitry- or cell-specific manipulations to target mRNA and/or proteins should be utilized.

There is growing appreciation that combining multiple genetic mutations or several environmental factors in a single model could be more informative. Recent studies from John Waddington's and Urs Meyer's groups are an intriguing example of how one can proceed with more complex but etiologically relevant models of not only G x E but G x G and E x E interactions (118, 119). In addition to conventional breeding approaches, newer technologies include plasmid-based cell-type-specific and inducible expression systems using *in utero* gene transfer by targeting multiple genes (120). A complementary methodology is to suppress the expression of target genes using RNA interference

(RNAi) knockdown technology (121). Several susceptibility genes can be knocked down simultaneously in mice carrying multiple siRNA expression transgenes.

Most studies have focused on neuronal functions of susceptibility genes. However, these genes are also expressed by glial cells (122, 123). Given, growing interest in the role for glia cells in mediating the effects of stress and microbial pathogens, GEI models with cell-specific perturbation of candidate genes are also needed. A recent study has provided the first evidence for the potential role of DISC1 in astrocytes, connecting DISC1 and serine racemase in modulating NMDA receptor functions (124).

In conclusion, GEI animal models have already begun to provide new insights into the etiological complexity and heterogeneity of schizophrenia. We believe GEI animal models will continue to be a crucial tool to advance our knowledge about this debilitating disease and help searching for new treatment options.

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CHAPTER 3:

***Toxoplasma gondii* and cognitive deficits in schizophrenia:**

an animal model perspective

(published in *Schizophrenia bulletin*, 2012)

Abstract

Cognitive deficits are a core feature of schizophrenia. Epidemiological evidence indicates that microbial pathogens may contribute to cognitive impairment in patients with schizophrenia. Exposure to *Toxoplasma gondii* (*T. gondii*) has been associated with cognitive deficits in humans. However, the mechanisms whereby the parasite impacts cognition remain poorly understood. Animal models of *T. gondii* infection may aid in elucidating the underpinnings of cognitive dysfunction. Here, we (i) overview the literature on the association of *T. gondii* infection and cognitive impairment, (ii) critically analyze current rodent models of cognitive deficits resulting from *T. gondii* infection, and (iii) explore possible mechanisms whereby the parasite may affect cognitive function.

3.1 Introduction

Cognitive impairment is an important feature and a serious problem in patients with schizophrenia. As many as 85% of patients exhibit some degree of cognitive dysfunction (50). While cognition encompasses many mental processes, seven domains are consistently impaired in schizophrenia: speed of processing, attention, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving, and social cognition. Three types of learning and memory that are severely affected are spatial, olfactory, and associative (125). Dysfunction can be present prior to the onset of psychosis, but more typically is observed concomitantly with positive symptoms. Unfortunately, treatment with anti-psychotics that can ameliorate positive symptoms do not significantly improve cognitive functioning (51). As the underlying mechanisms of cognitive impairment in patients with schizophrenia are not completely understood, the development of better therapies is impeded.

Recently, there has been a growing interest in the role of infectious agents in the development of psychiatric disorders. Epidemiological and immunological clinical studies have identified microbial factors that may contribute to cognitive impairment in patients with schizophrenia. Herpes Simplex 1, Cytomegalovirus, influenza and the protozoan *Toxoplasma gondii* (*T. gondii*) have all been implicated in the development of memory deficits in both non-schizophrenic and schizophrenic individuals (126, 127). The role of *T. gondii* exposure in schizophrenia related cognitive dysfunction is interesting in that while all other candidate pathogens are viruses, *T. gondii* is thus far the only implicated protozoan. The parasitic infection may contribute to cognitive impairment by

affecting various brain systems, including glutamate (GLU) synaptic neurotransmission. Indeed, the role of GLU in schizophrenia and cognition has been extensively studied and is an important line of research. However, we still know very little about the exact mechanisms whereby *T. gondii* infection leads to cognitive impairment. Animal models of *T. gondii* infection can help advance our understanding of these mechanisms and facilitate identification of novel therapeutic targets.

In this chapter we will (i) overview the literature on the association of *T. gondii* and cognitive impairment, (ii) critically analyze current rodent models of cognitive deficits resulting from *T. gondii* infection, and (iii) explore the possible mechanisms whereby the parasite may affect cognitive function. We will propose future directions in translational research with animal models of *T. gondii* infection.

3.2 *T. gondii* infectious cycle

T. gondii is an intracellular protozoan parasite that infects approximately a third of the human population (128). The three known clonal lineages of the parasite (Type I, II, and III) differ in their virulence, dissemination pattern, growth rate, immune response activation, and prevalence (10). Despite such differences, the three stages of *T. gondii*'s infectious cycle are common for all strains.

In all species, the parasite starts its cycle of replication and dissemination with the tachyzoite stage. This parasite stage is present during acute infection, whereby tachyzoites actively invade any nucleated cell in the body, replicate asexually, and egress

to infect other cells, destroying the host cell in the process. The second stage is noted by the localization of bradyzoite tissue cysts in skeletal muscle and most notably, the brain. The dormant tissue cysts are present in all hosts and are characteristic of chronic infection. The third stage occurs only in the feline intestinal tract, and is characterized by the production of oocysts through sexual reproduction of the parasite. The infectious oocysts are excreted into the environment, where they can be transmitted to other organisms (2). Converging evidence indicates that *T. gondii* manipulates rodent behavior to facilitate the parasite's transmission to the feline host. The behavioral effects of the parasite do not seem to be related to non-specific changes observed in sick animals but in fact suggest that the entire behavioral repertoire is affected to make an infected host a more likely prey for felines (128).

Oocysts and cysts can be transmitted to humans in a number of ways, including consuming undercooked meat infected with cysts, drinking water contaminated with oocysts, or handling soil contaminated with oocysts. When oocysts or tissue cysts are consumed, the parasite converts into actively invading and quickly replicating tachyzoites. Under surveillance and inhibitory control by the host immune system, tachyzoites enter into the dormant tissue cyst stage. However, tissue cysts are capable of rupturing, with bradyzoites transforming back into tachyzoites (2).

In humans, the transition of bradyzoites back to tachyzoites in the brain can have serious health consequences for immune compromised individuals (e.g. HIV-infected patients). The weakened host immune system cannot control the parasite from continually

invading and destroying brain cells, which results in toxoplasmic encephalitis (2). As encephalitis is not observed in healthy people, it has long been thought that *T. gondii* infection only harms immune-compromised patients. However, it is becoming evident that *T. gondii* infection can also cause problems in immune competent individuals. Indeed, infection has been associated with a number of behavioral changes, including cognitive deficits.

3.3 *T. gondii* and cognitive deficits in humans

T. gondii exposure has been associated with cognitive impairment in non-schizophrenic and schizophrenic subjects. One of the first studies to evaluate the role of *T. gondii* infection in cognitive impairment was published in 1953 by Burkinshaw et al. They found that 89% of *T. gondii* seropositive patients at a mental institution had severe cognitive impairments, with an intelligence quotient (I.Q.) below the normal range (<70). However, they could not definitively attribute the mental defects to toxoplasmosis(128). Similarly, following a group of 24 children congenitally infected with *T. gondii*, Wilson et al. determined 4 (17%) of them had severe cognitive impairments (I.Q. range 36 – 62), while 6 (25%) showed a drop in intelligence over time (I.Q. score of 97 down to 74) (129).

A few studies have associated *T. gondii* infection with less severe cognitive changes. In 1973, Saxon et al revealed that children who were congenitally infected with *T. gondii* had significantly lower I.Q. scores (93.2) as compared with their age matched uninfected controls (109.8) (130). Similarly, results from the Otis questionnaire conducted by Flegr et al revealed that young adult men (age 19-21) infected with *T. gondii* have lower verbal

intelligence as compared with their uninfected counterpart (129). Likewise, infected young adult men and the elderly (age 19-60) showed greater impairment in delayed and immediate memory as compared with uninfected controls (129).

One caveat of these studies is that the exact time of infection is unknown. As has been reviewed in detail by Jones et al, during pregnancy the risk of vertical transmission and severity of cognitive symptoms in offspring vary across the trimesters. For instance, while there is only a 10-25% risk of vertical transmission during the first trimester, exposure at this stage leads to mental retardation in offspring. In contrast, despite the fact that women who acquire *T. gondii* during the third trimester have a 60-90% risk of passing the parasite to the fetus, infection during this developmental stage produce minimal cognitive impairment in offspring(3). While the study of Saxon et al. provides evidence for subtle cognitive changes due to congenital *T. gondii* infection, it is possible that infection during other life stages (e.g. puberty) may cause similar outcomes. Such information can be useful in the development of preventive medication. For instance, the prospective study performed by Saxon et al, included a group of congenitally infected children who were treated with sulfadiazine and pyrimethamine for the first month after birth. The I.Q. of this group at 2-4 years of age was comparable with age-matched controls, while that of the untreated group was significantly lower(130).

Studies on the effects of *T. gondii* infection on cognitive dysfunction in people with schizophrenia are inconclusive. One study found no significant association between *T. gondii* infection and cognitive deficits in patients (aged 13-75 years old), although

seropositive subjects performed poorer on the Trail Making Test (TMT) than did seronegative patients. The age at which the individuals were first exposed to *T. gondii* was not mentioned (126). In contrast, Brown et al. reported that patients prenatally exposed to *T. gondii* exhibited worse executive functioning and memory than unexposed schizophrenics (131). This was evidenced by the increased number of errors made on the Wisconsin Card Sorting Test, and the greater length of time taken to complete part B of the TMT. As the study conducted by Brown et al. also included individuals prenatally exposed to influenza, it is difficult to unequivocally conclude that *T. gondii* infection contributed to cognitive dysfunction in individuals with schizophrenia. It is also unclear whether the cognitive deficits in infected patients are more severe than in seropositive controls. Although humans are not intermediate hosts for the parasite, memory or attention abnormalities in infected individuals might be a “side-effect” of parasite manipulation as discussed in detail elsewhere (128). As there is likely significant resemblance in the mechanisms whereby *T. gondii* impacts the brain and behavior of different species (128), rodent models will help to better understand how *T. gondii* infection contributes to cognitive deficits in infected patients.

3.4 Cognitive abnormalities in *T. gondii*-infected rodents

Cognitive effects of *T. gondii* have been evaluated in tests for spatial, olfactory, and associative learning and memory (Table 3.1).

Table 3.1 The Effect of *T. gondii* Infection on Learning and Memory in Rodents

F= female; M= male

Test	Species	Sex	Finding	Ref
Spatial Learning and Memory				
Labyrinth	Mouse, Rat	F	Impaired Learning	(132)
	Mouse, Rat	F	Impaired Learning	(133)
	Mouse	F	Impaired Memory	(133)
	Rat	F	No Impaired Memory	(133)
Y-maze	Mouse	F	Impaired Working Memory	(134)
	Mouse	F	No Impaired Recognition Memory	(134)
	Mouse	F, M	No Impairment	(135)
	Mouse	M	No Impairment	(136)
Radial Arm Maze	Mouse	F	Impaired Recognition Memory	(137)
Morris Water Maze	Rat	M	No Impairment	(138)
Object Recognition	Mouse	M	No Impairment	(139)
Olfactory Based Learning and Memory				
Social transmission of food preference	Mouse	F	No Impairment	(138)
	Mouse	F	No Impairment	(140)
	Mouse	M	Impaired Learning and Memory	(141)
Associative learning and memory				
Passive avoidance	Mouse	N/A	Impaired Memory	(142)

3.4.1 Spatial Learning and Memory

Spatial learning and memory require the processing of external cues to complete a task.

This is mainly studied in rodents through the use of mazes. The earliest reports on changes in spatial learning and memory due to *T. gondii* infection come from the studies in the late 1970s. Using labyrinth test, Piekarski et al found that chronically infected adult female mice and rats showed impaired learning as compared to uninfected controls (132). A year later, Witting's study further demonstrated that *T. gondii* infection impaired learning in both acute and chronically infected adult female mice and rats. Learning impairment was observed as early as 2 days post infection (acute) and as late as 6 weeks post infection (chronic). Notably, memory was found to be affected differently between the two species, with only infected mice exhibiting severe memory impairment (133).

Although the spatial learning and memory studies using labyrinth tests provided consistent and intriguing results, this task is no longer popular. Now, Y maze, radial arm maze or Morris water maze are commonly employed. Y maze is a useful tool for studying spatial working (short-term) and recognition (long-term) memory. Using Y maze, Kannan et al found that chronic infection of young adult Balb/C female mice (9 wks old) led to deficits in spatial working memory, but not spatial recognition memory. Curiously, while the effects of two Type II strains of *T. gondii* were compared, Pru and ME49, only infection with ME49 was associated with impairment of spatial working memory (134). Neither parasite strain produced deficiency in spatial recognition memory in the infected female mice, as determined by the similar amount of time control and infected groups spent exploring the novel arm (134). In a different study, mice were exposed to *T. gondii*

at one of three time points: (i) 8 weeks of age, (ii) congenitally by acutely infecting pregnant dams, or (iii) congenitally through mating of chronically infected females (135). It was found that male and female strain A albino mice, exposed at all three time points to the Beverley Type II strain of parasite, preferred the familiar arm over the novel one, while controls expectedly preferred the novel arm. Intriguingly, the groups exposed congenitally to the parasite spent more time in the familiar arm as compared with adult acquired infection group. The outcome was interpreted as *T. gondii*-induced neophobia (135). The increased neophobia in infected mice appears different from the behavioral responses seen in rats, although the rat studies used a different paradigm (143). Adult infected male mice demonstrated deficits in spatial recognition memory measured as lack of preference for either arm of Y maze (136).

Radial 8-arm maze is also widely used to study spatial working and recognition memory. The increased number of arms compared to the Y maze adds complexity to the rodent's task, helping to uncover more subtle deficits in learning and memory. One study with *T. gondii* infected rodents used radial arm maze to assess spatial recognition memory only. This task requires rodents to remember the maze arm with food rewards, in order to reach the food more quickly on following days. Compared to uninfected controls, chronically infected adult female mice (10 weeks of age) of a mixed Balb/C:C57BL/6 background spent more time searching for the food reward (137), suggesting a deficit in spatial recognition memory. Notably, this deficiency was not seen in chronically infected female mice in Y maze (134).

Morris water maze (MWM) is another task to evaluate spatial learning and memory. As with Y and radial arm mazes, the MWM protocol can be modified to assess both spatial working and reference memory. Using MWM, Vyas et al evaluated the effects of *T. gondii* infection on spatial memory in rats (138). They found that control and chronically infected male rats spent the same amount of time in the quadrant that previously contained the escape platform, indicating no deficit in spatial memory (138). Spatial recognition memory in infected rodents was also examined in object recognition and spatial placement tests. It was determined that chronic infection of 8-week-old C57BL/6 male mice did not affect cognitive functions in these tests (139).

3.4.2 Olfaction based Learning and Memory

The social transmission of food preference (STFP) test is based on olfaction and represents another type of cognitive test used in infected rodents. This task requires intact olfaction and sufficiently high levels of social non-aggressive interactions. Two groups used STFP to evaluate cognitive effects of chronic *T. gondii* infection in young adult Balb/C mice. Both studies found that *T. gondii* infection did not affect learning and memory in female mice as evidenced by their consuming greater amounts of “familiar” food (the one that was previously presented by a social partner) compared to “novel” one (138, 141). In contrast, memory deficit was observed in infected males, as evidenced by consumption of similar amounts of both types of food. Notably, gender-dependent effects of *T. gondii* on olfaction-related behaviors have also been documented in human studies. Flegr et al. found that cat odor attractiveness increased for infected men while decreased for infected women (15).

3.4.3 Associative Learning and Memory

Associative learning and memory can be studied in active and passive avoidance tests. To date, only one study has evaluated the effect of congenital exposure to *T. gondii* on passive avoidance in adult mice. In this study, mice were exposed to *T. gondii* at embryonic day 5, 10, and 15. It was found that congenital infection during early and intermediate gestation (5 and 10 days respectively) but not late gestation (15 days) leads to impaired passive avoidance in adult mice. This was demonstrated by a shorter latency time to enter the dark chamber, as well as a greater number of entries into the dark chamber (142).

The inconsistent results of the rodent studies can be explained by experimental differences, including rodent sex, species, and strain (144). For example, direct effects of *T. gondii* on sex hormone production and the role of sex hormones in susceptibility to infection were shown to influence sex-related cyst burden and temporal differences in cytokine production in the brain (145, 146). Such differences can contribute to sex-dependent behaviors, emphasizing the importance of testing male and female animals. With regard to species-specific differences, milder cognitive abnormalities were reported in rats compared to mice. The variable outcomes could be explained by differential resistance between two species. Unlike mice, rats are less susceptible to *T. gondii* infection and exhibit the clinical course and *in utero* transmission that may better mimic the human situation (144). Notably, greater resistance in rats appears to be related to later stages of infection as the early dissemination of parasites has been found to be very similar between species (147). However, it remains unclear how species-specific brain

physiology and behavioral biology can contribute to variable cognitive deficits produced by *T. gondii*.

3.5 *T. gondii* effects on the brain

Numerous studies have evaluated the molecular, cellular and immune effects of *T. gondii* on the brain. In vitro experiments have demonstrated that tachyzoites infect and form cysts within microglia, astrocytes, and neurons (146). Activated microglia and astrocytes limit parasite replication and spread by stimulating expression of the NF-kB family of transcription factors to regulate production of pro- and anti-inflammatory cytokines (146). In turn, cytokines (e.g. IL-1, IL-6, and TNF-alpha) can influence synaptic neurotransmission and potentially contribute to cognitive deficits (148).

In addition to the effects of immune activation (e.g. indirect mechanisms), it has been recently shown that the parasite itself is able to affect dopamine neurotransmission in the brain (e.g. a direct mechanism). McConkey and his colleagues in a series of elegant experiments have demonstrated that the parasite expresses homologs of enzymes involved in the dopamine synthesis pathway: phenylalanine and tyrosine hydroxylase (149). *In vitro* work with PC12 cells has demonstrated that *T. gondii* cysts can increase cellular release of dopamine (20). Additionally, *in vivo* work has shown that parasite cysts can synthesize dopamine, and that infection increases whole brain dopamine levels in infected mice (150). Yet, a recent study reports no changes in monoamine content in the frontal cortex and striatum of congenitally infected CD-1 mice (151). Future studies are clearly needed to further evaluate alterations in dopamine neurotransmission in

infected rodents *in vivo* and link these changes to behavioral abnormalities, including cognitive impairment. However, it seems less likely that only dopamine changes will be able to explain the entire spectrum of behavioral responses in infected animals. It seems that more attention should be paid to glutamate (GLU) neurotransmission, alterations in which have been strongly associated with cognitive dysfunction in schizophrenia.

Administration of non-competitive *N*-Methyl-D-aspartic acid receptor (NMDAR) antagonists, phencyclidine (PCP) and ketamine, to non-psychotic individuals can lead to behavioral changes that mimic positive, negative, and cognitive symptoms, including alterations in attention, memory, and reasoning. Subsequent studies using NMDAR antagonists, brain imaging, human postmortem samples, genetic approaches, and pre-clinical animal models have provided further evidence of the importance of GLU and its receptors in cognitive functioning (152). These studies have suggested that dysfunction in NMDAR may be responsible for aspects of cognitive deficits in people with schizophrenia. However, it has been recently proposed that hyper function of NMDA receptors mediated through activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors can also contribute to the pathophysiological mechanisms of cognitive impairment in schizophrenia (152).

The link between *T. gondii* and changes in GLU neurotransmission remains poorly understood. Up-regulation of a glutamate receptor antagonist has been mostly evaluated. An endogenous metabolite of tryptophan metabolism and a NMDA receptor antagonist, kynurenic acid (KYNA), has been hypothesized to be a pathogenic link between *T.*

gondii infection and cognitive impairment in schizophrenia (153). Elevated levels of KYNA can lead to excessive stimulation of the NMDAR, affecting GLU neurotransmission and resultant cognitive function (153). Thus, diminishing elevated KYNA levels is predicted to ameliorate cognitive deficits. Knockout mice with deletion of the enzyme that converts kynurenine into KYNA, kynurenine aminotransferase II, have lower levels of KYNA and perform better in cognitive tests compared to control mice (154). As rodents infected with *T. gondii* and patients with schizophrenia have increased brain levels of KYNA (153), one could predict that reduction of levels of this NMDA antagonist may have therapeutic effects.

The GLU system can also be affected by the host immune system's response to *T. gondii* infection via secreting cytokines (e.g., IL-6 or TNF-alpha)(148). Another immune mechanism of GLU synaptic dysfunctions may be related to production of anti-NMDA receptor antibodies. Indeed, recent studies have implicated autoantibodies to the NMDA receptor in the causation of cognitive deficits (155). While it remains to be seen whether such autoantibodies are produced by *T. gondii* infection, it has been reported that the infection-induced IgG antibodies are able to cross-react with neural epitopes, including the NMDAR (156). It is also possible that the immune response to the parasite could alter numbers of NMDAR similar to the decreased expression of NMDAR due to prenatal infection with cytomegalovirus (157). In addition, *T. gondii* could impact NMDAR functioning by affecting major histocompatibility complex (MHC) signaling (158). Indeed, *T. gondii* infection has been shown to decrease neural MHC II expression (159), which has been associated with cognitive deficits in CD4 KO mice (160). Collectively,

these studies suggest that the host immune response could affect GLU neurotransmission, resulting in cognitive impairment in infected individuals. However, more studies are clearly needed to provide a better understanding of the underlying molecular pathways.

3.6 Future directions

Rodent models of cognitive dysfunction associated with *T. gondii* infection disease are important for advancing our knowledge of the molecular pathogenesis and the development of therapeutics. However, there are still several lines of work that need to be improved. In addition to schizophrenia, cognitive impairments are associated with other disorders, including obsessive-compulsive, bipolar disorder and Alzheimer's disease (161, 162). It is possible that there is substantial commonality in the pathophysiology of cognitive dysfunction in all these illnesses. Therefore, to create a more comprehensive rodent model of cognitive impairment associated with schizophrenia, it is necessary to model other schizophrenia-like behavioral changes as well (163).

Given that microbes probably interact with genetic factors to contribute to psychiatric disease, there is a need in developing animal models based on interactions between *T. gondii* infection and candidate genes, e.g., Disrupted in Schizophrenia 1 (*DISC1*). *DISC1* was first identified in a balanced chromosomal translocation (1:11) (q42.1; q14.3) that segregates in a Scottish pedigree with major mental disorders (164). *DISC1* is located within a region of the human genome identified as likely to harbor a susceptibility gene for schizophrenia, mood disorders and autism spectrum disorders in individuals who do not carry the translocation. Numerous investigations have implicated *DISC1* and

interacting partners in neurodevelopment, adult neurogenesis in the hippocampus and synaptic neurotransmission in adulthood (165). In the context of this chapter, it has been shown that prenatal immune activation induced by poly (I:C) to mimic viral infection produces the behavioral abnormalities previously unseen in mutant DISC1 mice without prenatal challenge (94). Therefore, combining genetic mutations and *T. gondii* infection may better model the pathogenesis of cognitive impairments in schizophrenia.

One needs to take into account timing of infection. Prenatal and early postnatal models of *T. gondii* infection mimic different human conditions. Prenatal infection in rodents would be related to a situation where circulating anti-parasitic antibodies in the blood of a pregnant woman may be potentially pathogenic due to cross-reactivity with the fetal brain (166). In contrast, early childhood *T. gondii* infection seems to be best modeled by pre-adolescent infection in rodents to affect postnatal development of cortical areas involved in cognitive functions (165). A quite different pathophysiological mechanism (e.g., altered neurotransmission) could be modeled by infection of adult animals.

Last but not the least, new insights into the mechanisms of cognitive impairments in schizophrenia will be obtained with pharmacological studies similar to the one pioneered by Webster and her associates who treated the behavioral abnormalities in infected rats with antipsychotics and mood-stabilizer medications (8). Further work is clearly needed in this direction with existing and new anti-parasitic drugs, cognitive enhancers and compounds targeting dopamine and glutamate neurotransmission.

3.7 Conclusions

Available data suggest that *T. gondii* infection may contribute to cognitive deficits in people with schizophrenia. Such abnormalities may be subserved by different mechanisms, including alterations in GLU synaptic neurotransmission. However, there is a need to generate more sophisticated animal models of infection-induced cognitive dysfunction, likely through a combination of genetic and environmental factors, taking into account timing of infection and use of pharmacological approaches to further our understanding of the pathophysiology of cognitive abnormalities in schizophrenia. All such work will hopefully facilitate the development of novel therapeutics to aid those suffering from this devastating disease.

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CHAPTER 4:

Toxoplasma gondii strain-dependent effects on mouse behaviour

(published in *Folia Parasitologica*, 2010)

Abstract

Toxoplasma gondii (*T. gondii*) reportedly manipulates rodent behavior to increase transmission to its definitive feline host. We compared the effects of mouse infection by two Type II strains of *T. gondii*, Prugniaud (PRU) and ME49, on attraction to cat odor, locomotor activity, anxiety, sensorimotor gating, and spatial working and recognition memory 2 months post-infection (mpi). Attraction to cat odor was reassessed 7 mpi. At 2 mpi, mice infected with either strain exhibited significantly more attraction to cat odor than uninfected animals did, but only PRU-infected mice exhibited this behavior 7 mpi. PRU-infected mice had significantly greater body weights and hyperactivity while ME49-infected mice exhibited impaired spatial working memory. No differences in parasite antibody titers were seen between PRU- and ME49-infected mice. The present data suggest the effect of *T. gondii* infection on mouse behavior is parasite strain-dependent.

4.1 Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite found worldwide. It infects nucleated cells of all mammals, including humans. However, it requires a cat host for sexual reproduction. Therefore it has been suggested that *T. gondii* manipulates rodent behavior to increase transmission efficiency (128).

Rodents have an innate fear of cat odors (167). Infection with Type II *T. gondii*, Prugnnaud (PRU) and ME49, has been shown to decrease avoidance of cat odor and increase attraction to it without significantly affecting general anxiety and fear, suggesting specificity in the type of manipulation by the parasite (138, 168, 169). Many studies on the behavioral effect of *T. gondii* infection have focused on the early latent phase (e.g., 4-10 weeks post-infection), after bradyzoite establishment in the central nervous system (137, 138, 170-172). Work on behavioral manipulation later in the latent phase of the parasite (e.g. >5 months post-infection) is limited (173). Although the behavioral effects of PRU and ME49 have been studied before, a direct comparison of these strains has not been reported.

The aim of this study was to directly compare the effects of latent infection of two strains of Type II *T. gondii*, PRU and ME49, on mouse behavior. We hypothesized that manipulation effects for direct cat transmission (e.g. cat odor attraction) would be similar between strains, while behaviors not directly associated with transmission to cats could be differentially affected. To this end, attraction to cat odor, open field activity, anxiety, pre-pulse inhibition (PPI) of the acoustic startle, and spatial working and recognition

memory were tested in mice 2 months post-infection (mpi). In addition, to evaluate duration of manipulation for direct transmission, we reassessed attraction to cat odor 7 mpi.

4.2 Materials and Methods

4.2.1 Animals

Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animal protocols were reviewed and approved by the Animal Care and Use Committee of Johns Hopkins University (JHU). Mice were housed 5 per cage in the JHU animal facility with 14.5/ 9.5 hours of light/dark cycle and free access to food and water.

4.2.2 *Toxoplasma gondii* Culture and Infection

Prugniaud (PRU) and ME49 tachyzoites were maintained by passage in human foreskin fibroblast monolayers. Tachyzoites were released from fibroblasts by using, in succession, 18-, 23-, and 27-gauge needles. Parasites were separated from cell debris by filter sterilization (Polycarbonate Membrane Filter, Whatman) and resuspended in Dulbecco's phosphate buffered saline (DPBS). Mice (9 weeks old) were either mock-infected with sterile DPBS or infected with 400 tachyzoites (2 parasites/ μ L) intraperitoneally. All behavioral studies were performed on the same cohort 2 mpi, and only aversion to cat odor was reassessed 7mpi.

4.2.3 Infection Determination

Infection was confirmed via serology as previously described (174). Briefly, sera were collected from mice 9 mpi and IgG antibodies to *T. gondii* measured by microplate enzyme immunoassay.

4.2.4 Aversion to Domestic Cat Urine

Mice were habituated to an open-field box (48cm X 45cm X 21cm) for 5 min daily for three consecutive days prior to testing. On testing day, 50-mL vial caps containing either 1-mL of undiluted domestic cat (Johns Hopkins University School of Medicine Deaf cat colony) or domestic dog (a pet) urine were placed in diagonal corners. Mice were placed in the centre and allowed to freely explore for 10 min while being videotaped. The position of the odors was altered between each mouse to avoid positional bias. Time spent near each odor was recorded using Stopwatch+ (Centre for Behavioral Neuroscience) by individuals blind to the type of odor. Occupancy was calculated as the percent time spent near each odor for the first 5 min of the 10-min trial to evaluate early responses to cat odor. Other behavioral tests were run as previously described (72) and explained briefly below.

4.2.5 Novelty-Induced Activity

Novelty-induced activity in the open field was assessed over a 30-min period using activity chambers with infrared beams (San Diego Instruments Inc., San Diego, CA, USA). Horizontal and vertical activities, stereotypic activities, and time spent in the center or along the walls (thigmotaxis) of the chamber were automatically recorded.

4.2.6 Spontaneous Alteration and Spatial Recognition Memory

Y-maze consisting of three arms of equal length, interconnected at 120°, was used as described (175). The test included two sessions, which evaluate spatial working and recognition memory, respectively. In the first session we scored the number of alternations done by the mouse when all three arms are visited, without entering the same arm twice in a row. After 5-7 days, the second session was performed and consisted of two trials. During trial 1, one maze arm was blocked and the mouse allowed to freely explore the other two arms for 5 min. Trial 2 began after a 30-min delay. During trial 2, the mouse was allowed to freely explore all three arms for another 5 min, taking advantage of the innate tendency of mice to explore novel unexplored areas (e.g., the previously blocked arm). Mice with intact recognition memory prefer to explore novel over familiar arms, whereas mice with impaired spatial memory enter all arms equally. Motor activity was recorded as number of arms visited for the whole duration of the test. Time spent in the blocked arm during the first 2 min of the 5-min test period was used to assess spatial memory in mice as the first 2 min of testing have been shown to be most sensitive to manipulations (175).

4.2.7 Anxiety

The elevated plus maze is a cross of two arms that are open to the environment and two arms that are enclosed by side and end walls. The arms are connected by a central area. The maze is elevated from the floor (75 cm). Mice were placed in the central area of the maze facing a closed arm. Explorative behavior was recorded via a video camera. The number of open- and closed-arm entries and the time spent in both types of arms were

scored. An entry was defined as all four paws in one arm. Anxiety was scored as the percentage of time spent in the open arm in relation to the total 5-min test.

4.2.8 Sensorimotor Gating

Pre-pulse inhibition of the acoustic startle (PPI) was used to assess sensorimotor gating. Mice were acclimatized in startle chambers (San Diego Instruments, Inc., San Diego, CA) to a 70-dB background noise (continuous throughout the session) for 5 min, followed by the presentation of ten 40 ms 120 dB white noise stimuli at 20 s inter-stimulus intervals (habituation). After habituation, mice were left in the enclosure for 5 min without presentations of any startle stimuli. Immediately after, the pre-pulse inhibition (PPI) session was begun, consisting of six presentations of each trial type in a pseudorandom order. The types of trials include: pulse-alone (120-dB, 100-msec, broadband burst), omission of stimuli, and four pre-pulse–pulse combinations consisting of a 20-msec pre-pulse broadband burst at either: 74, 78, 82, 86 or 90 dB, presented 80-msec before the pulse. Startle reactivity and PPI were evaluated by separately comparing absolute values of the startle amplitudes for different types of trials between three groups. Compared to using percentage values of PPI, the absolute values approach provides a better evaluation of startle reactivity both at pulse-alone and pre-pulse trials (176).

4.2.9 Statistical Analysis

Data were tested for normality and if failed were subjected to rank transformation. Afterwards, data were analysed using ANOVA with strain of the parasite and time (when applicable) as independent variables. In addition, planned Student t-test was used to

evaluate cat attraction between the two strains of parasite, and $p < 0.05$ was considered significant.

4.3 Results

We found that both strains of parasite caused comparable cat odor attraction in infected mice 2 mpi, but had a differential effect 7 mpi (Fig 4.1). At 2 mpi, compared to control mice, infected mice spent significantly more time near the cat odor. In addition, infected mice spent more time near the cat odor than dog odor (Fig 4.1A). At 7 mpi, PRU-infected mice continued to spend more time near cat odor than dog odor, as compared to controls (Fig 4.1B). In contrast, ME49-infected mice now spent comparable time near dog and cat odors, similar to control mice.

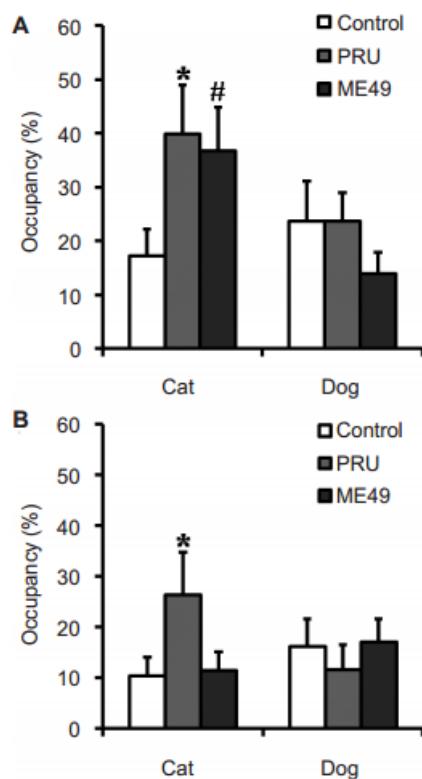


Fig 4.1 PRU- and ME49-infection increases mouse attraction to cat odor during the first 5 min of the 10-min trial

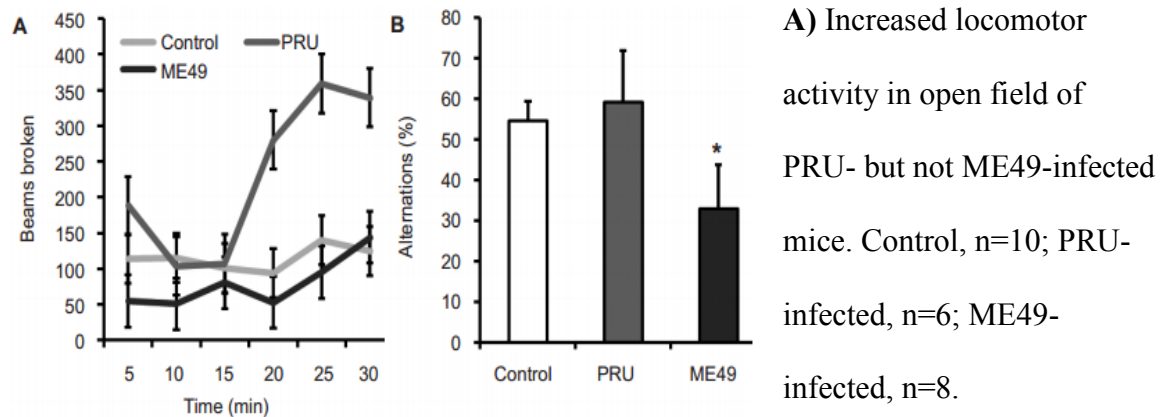
A) Infection increased occupancy near the cat odor 2 mpi. Control, $n=10$; PRU-infected, $n=6$, * denotes $p = 0.032$ relative to control; ME49-infected, $n=8$, # denotes $p = 0.05$ relative to control.

B) Increased occupancy near cat odor 7 mpi. Control, $n=10$; PRU-infected, $n=6$, * denotes $p = 0.066$ relative

to control; ME49-infected, $n=8$. Error bars denote SEM.

Although the effects of the two parasite strains were similar on cat attraction behavior 2 mpi, behaviors not directly associated with manipulation were differentially affected by the two strains of the parasite (Fig 4.2). PRU-infected mice had significantly heavier body weights than controls and ME49-infected mice [a group effect, $F(2, 25) = 4.77$, $p = 0.018$], which was abated at 7 mpi [a group effect, $F(2, 25) = 1.32$, $p = 0.287$] (Table 1). PRU-infected mice also showed increased locomotor activity in the open field, as evidenced by the greater number of broken beams compared to control (Fig 4.2A). In contrast, only ME49-infected mice exhibited decreased spontaneous alterations in the Y-maze, indicative of impaired spatial working memory (Fig 4.2B).

Fig 4.2 Differential effects of PRU- and ME49-infection on mouse behavior



B) Impaired spatial working memory in ME49- but not PRU-infected mice. Control, n=10; PRU-infected, n=6; ME49-infected, n=8, * denotes $p = 0.056$ relative to control (Student's *t*-test). Error bars denote SEM.

No effect of either parasite strain was found on spatial recognition memory, anxiety, or sensorimotor gating (Fig 4.3). There was no difference in spatial recognition memory between groups as evidenced by similar exploration time of the blocked arm during the first 2 minutes of testing [a group effect, $F(2, 25) = 1.0$, $p = 0.380$] (Fig 4.3A). No differences in anxiety were noted between control and either infected group [a group effect, $F(2, 25) = 0.68$, $p = 0.517$] (Fig 4.3B). Finally, control and mice infected with either parasite strain had similar levels of the acoustic startle of the PPI (Fig 4.3C).

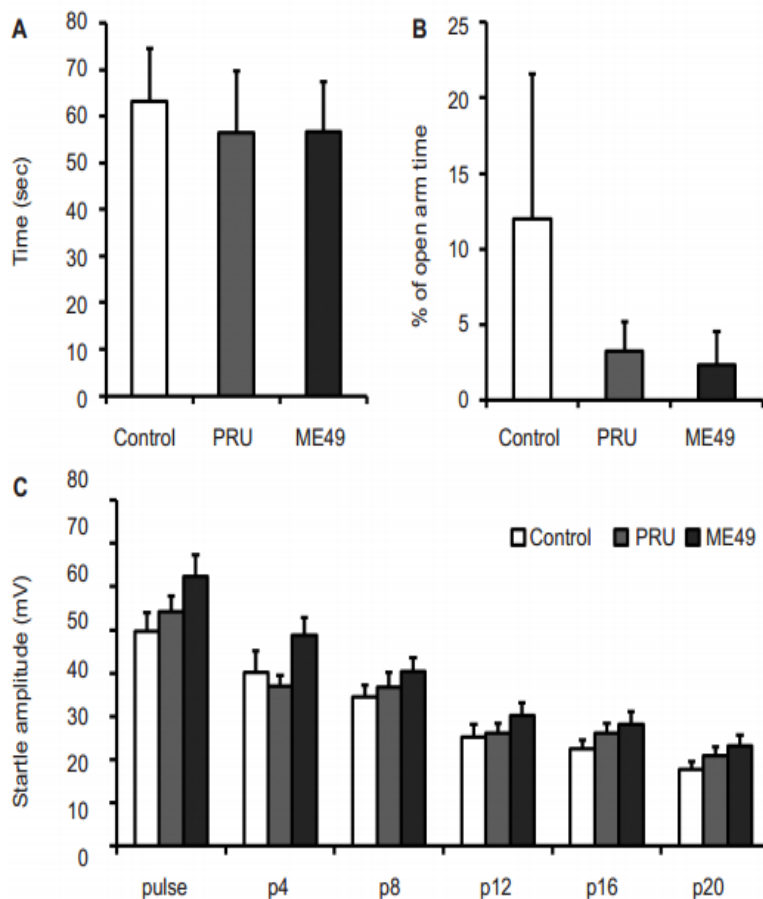


Fig 4.3 No effect of infection on spatial recognition memory, anxiety, or sensorimotor gating

A) No difference in spatial recognition memory between PRU- and ME49-infected mice and controls. Control, $n=10$; PRU-infected, $n=7$; ME49-infected, $n=9$.

B) No changes in anxiety between infected and controls. Control, n=10; PRU-infected, n=7; ME49-infected, n=9.

C) No difference in pre-pulse inhibition of acoustic startle between infected and controls. Control, n=10; PRU-infected, n=7; ME49-infected, n=9.

X-axis: p4, p8, p12, p16, and p20 are intensities of different pre-pulse trials above the background noise level (70 dB). Error bars denote SEM.

The serology results confirmed infection with PRU and ME49 [a group effect, $F(2, 15) = 1883$, $p < 0.001$] and found no differences in serum antibody titres between the two groups of infected mice [t-test, $p = 0.847$] (Table 4.1).

Table 4.1 Average body weight and *Toxoplasma gondii* antibody titres of infected and control mice

		Control	PRU	ME49
Body Weight (g)	2 mpi	25.8 ± 1.34	* 30.6 ± 1.35	25.1 ± 1.15
	7 mpi	27.3 ± 1.08	29.1 ± 1.77	26.3 ± 0.74
Antibody Titre (O.D.)	9 mpi	0.15 ± 0.06	* 3.77 ± 0.04	3.76 ± 0.03

PRU-infected mice weighted significantly more than control and ME49-infected mice 2 mpi. Control, n=10, PRU-infected, n=7; ME49-infected, n=9, * denotes $p=0.009$ relative to control and $p=0.015$ relative to ME49-infected mice. No differences in weights were seen 7 mpi.

High antibody titres were present in infected mice as compared to control. Control, n=3; PRU-infected, n=6; ME49-infected, n=7, * denotes $p < 0.001$. All data are mean values \pm SEM.

4.4 Discussion

The data support our hypothesis that both strains of the parasite comparably manipulate behavior associated with direct cat transmission and differentially manipulate behaviors not directly associated with feline transmission. However, while mice infected with either parasite demonstrated comparable attraction to cat odor at 2 mpi, only PRU-infected mice exhibited this behavior 7 mpi.

Our present results appear consistent with previous studies that have reported *T. gondii* infection in wild and laboratory rodents cause attraction to cat predator odor (138, 168, 169). Somewhat unexpectedly, our findings seem to suggest that duration of manipulation, or at least some behavioral manifestations of manipulation, might vary between strains of the parasite. Future work is clearly needed to further our understanding of possible permanent and transient aspects of the manipulation effects of *T. gondii*. Strain differences in behavioral manipulation may be due to differences in prolonged cyst burden. For instance, although at 1 mpi high PRU cyst density was found in many brain regions (138) only few ME49 cysts were observed in more chronic infections (144). Future studies will directly compare cyst number and localization of PRU and ME49 in

different brain regions and at different time points to determine whether this is in fact a factor in the transient manipulation of ME49.

Our data is also in line with studies that have shown infected mice display hyperactivity and learning and memory impairment (133). For example, studies on mice and rats with adult and congenital infection found hyperactivity in infected mice compared to controls between 2 and 7 mpi (177-180), similar to what we found with PRU-infected mice. In contrast, a study performed in infected mice and rats found decreased locomotor activity 2-3 mpi (133). Yet another work did not find any significant difference in activity between infected and control mice 3 mpi (171), consistent with our finding with ME49-infected mice. These discrepancies suggest strain-dependence in behavioral effects of *T. gondii*. Although these behavioral changes may not directly increase transmission of *T. gondii* to cats, both locomotor hyperactivity and impaired spatial working memory could potentially increase chances of being captured by a predator.

Similar to putative strain-dependent effects in cat attraction, behavioral effects not related to direct manipulation may be due to a number of factors. For instance, it is possible that cyst localization differs between strains. It has been shown that the amygdale of PRU infected mice contain high cyst density (138). It is conceivable that individual strains may have variable tropism to other brain regions, differentially affecting other behaviors than just innate fear. Behavioral differences may also be due to infection of different cell types. It is also possible that strains elicit varying immunological responses, resulting in dissimilar alterations in neurotransmission as the three clonal lineages of *T. gondii* differ

in their modulation of cytokine secretion (10). However, direct comparison of the effects of different strains within and between lineages on behavioral manipulation, neurobiology, and neuroimmunology need to be done to better understand mechanisms of behavioral effects caused by this parasite. Differential susceptibility of behaviors to parasite strains might partly explain the broad spectrum of behavioral abnormalities in animals and clinical manifestations in humans infected with *T. gondii* (5, 181).

In conclusion, our data indicate that infection with *T. gondii*, PRU and ME49, produced comparable attraction to cat odor in mice 2 mpi. We also found that the degree of attraction and the effects on other mouse behaviors may be influenced in a parasite strain-dependent manner.

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CHAPTER 5:

Sex-specific changes in gene expression and behavior induced by chronic

Toxoplasma infection in mice

(published in *Neuroscience*, 2012)

Abstract

There is growing evidence that *Toxoplasma gondii* modifies the behavior of its intermediate hosts. We investigated the molecular basis of these infection-induced behavioral changes, followed by five related behavioral tests to assess the extent of biological relevance. Gene expression signatures were generated in the frontal cortex of male and female mice during the latent stage of infection. We found marked sex-dependent expression differences in mice. In female mice, *Toxoplasma* infection altered the expression of genes involved in the development of the forebrain, neurogenesis, sensory and motor coordination (i.e., downregulation of fatty acid binding protein 7 and eyes absent homolog 1, upregulation of semaphorin 7A). In male mice, infection led mainly to modulation of genes associated with olfactory function (i.e., downregulation of a number of olfactory receptors and dopamine receptor D4, upregulation of slit homolog 1). While infection appears to affect the olfactory function in male mice, it is female but not male mice exhibited attraction to cat odor. In contrast, infected male mice showed a deficit in social transmission of food preference. In contrast to males, infected females displayed locomotor hyperactivity in open field. General olfaction and sensorimotor gating were normal in both male and female infection. Our results indicate that the sex of the host plays a major role in determining variable brain and behavior changes following

Toxoplasma infection. These observations are consistent with heterogeneity of neuropsychiatric outcomes of the infection in humans.

5.1 Introduction

Toxoplasma gondii is a common protozoan which infects humans and other animals.

Felines serve as the primary host in which *Toxoplasma* can undergo sexual reproduction and complete its life cycle. Humans, rodents, and other non-feline vertebrates can become infected with *Toxoplasma* and serve as intermediate hosts. After a short phase of acute toxoplasmosis, the infection proceeds into its latent phase, characterized by parasite cyst presence in the central nervous system and muscular tissue (182). The latent phase of infection, except in individuals with an immature or suppressed immune system, has been considered to be asymptomatic. Results of animal and human studies of personality profiles, behavior, and psychomotor performance have led to a reconsideration of this assumption (17, 134, 138, 144, 168, 174, 183, 184). For example, infected rodents show impaired learning and memory as well as increased activity (144). One of the most remarkable changes is that *Toxoplasma* infection can convert the rodents' natural aversion to feline odors into attraction (134, 138), presumably, in order to alter the behavior of the host to benefit the parasites' sexual cycle (144). In humans, persistent *Toxoplasma* infection has been associated with increased rates of serious psychiatric disorders, suicide, accidents, and reduced psychomotor performance (17, 174, 183, 184). Such behavioral changes induced by latent toxoplasmosis have been reported to differ depending on the sex of the host: males have shown lower rule-consciousness and greater jealousy, while females showed promiscuity and greater conscientiousness (18). The molecular mechanisms of altered behaviors in secondary hosts have not been elucidated. We have recently shown that infection of a human neural cell line with *Toxoplasma* tachyzoites (acute infection) results in the altered transcription of a number

of different genes associated with a wide range of biological functions (185). Fouts and Boothroyd (2007) have compared the gene expression profile in human foreskin fibroblasts (HFFs) infected with tachyzoites or bradyzoites for 48 hours and found no dramatic differences in the abundance of host mRNAs (186), although compared to tachyzoites, bradyzoites induced a lesser effect on genes involved in cytokine- and chemokine-signaling.

In this study, we examined the effects of persistent *Toxoplasma* infection on gene expression in the frontal cortex of male and female mice. In addition, in a separate cohort, we assessed behavioral abnormalities suggested to be changed by microarray analysis. The sex-dependent alterations in gene expression and mouse behaviors suggest the sex of the host plays an important role in the response to *Toxoplasma* infection.

5.2 Experimental Procedures

5.2.1 Animals

Male and female BALB/c mice (9 weeks old, The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animal protocols were reviewed and approved by the Animal Care and Use Committee of Johns Hopkins University (JHU). All experiments conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize the number of mice used and their suffering. Mice were housed 1-4 per cage (initially 5 per cage) in the JHU animal facility with 14.5/ 9.5 hours of light/dark cycle and free access to food and water.

5.2.2 *Toxoplasma* culture and infection

Prugniaud (PRU) strain was maintained by passage in human foreskin fibroblast (HFF) monolayers. Tachyzoites were released from cells by using, in succession, 18-, 23-, and 27-gauge needles. Parasites were separated from cell debris by filter sterilization (Polycarbonate Membrane Filter, Whatman) and resuspended in Dulbecco's phosphate buffered saline (DPBS). Mice of each sex were either mock-infected with sterile DPBS or infected with 400 tachyzoites (2 parasites/ μ l) intraperitoneally.

5.2.3 Sample preparation and microarray analysis

Surviving mice (infected female: n=5; infected male: n=5; female control: n=5; male control: n=4) were sacrificed approximately 6 month post infection and the frontal cortices harvested. At this time, sera were also collected and IgG antibodies to *Toxoplasma* measured by microplate enzyme immunoassay to confirm infection (Xiao et al., 2009). Total RNA was harvested using the RNeasy reagent (QIAGEN, Valencia, CA). The RNA concentration and integrity was determined using Agilent BioAnalyzer (Agilent, Santa Clara, CA). RNA transcript levels were quantified by microarray analyses. Briefly, total RNA was used to generate biotin labeled cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). Biotinylated cRNAs were purified, fragmented, and subsequently hybridized to Illumina Mouse Ref-8 v2.0 Expression BeadChips (Illumina, San Diego, CA). Each BeadChip has 24,000 well-annotated RefSeq transcripts with approximately 30-fold redundancy. The arrays were

scanned using an Illumina BeadStation 500X Genetic Analysis Systems scanner and the image data extracted using Illumina GenomeStudio software, version 3.0.

Data were analyzed using GeneSpring software v. 11.0.2 (Silicon Genetics, Redwood City, CA). Raw data were imported into GeneSpring and normalized using global normalization. The normalized data were used to identify changes in gene expression in the 2 group comparisons: male infected vs male control and female infected vs female control. For statistical analysis, one-way ANOVA was initially performed for each gene to identify statistically significant gene expression changes between the two groups, followed by Benjamini and Hochberg correction to control the false discovery rate. Genes whose transcripts with a FDR-adjusted p-value of < 0.02 and a fold change of > 1.7 in either direction were considered statistically significant and termed differentially expressed genes (DEGs). This cutoff was set based on previous success of verifying expression levels of genes at or above this level by real-time PCR (Cadet et al., 2010).

5.2.4 Data mining

Gene Ontology analysis was conducted for DEGs using the Spotfire analytic platform (<http://spotfire.tibco.com>). Selected genes were compared against the universe of unselected genes at each level of annotation and for each a p-value was calculated according to a hypergeometric distribution that represents the probability that the gene-functions are randomly distributed between groups (187). Pathway and function analyses were generated using Ingenuity Pathway Analysis (IPA, <http://www.ingenuity.com>,

Mountain View, CA) which assists with microarray data interpretation via grouping DEGs into known functions and pathways.

All microarray data obtained in this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) public repository, and they are accessible through GEO accession (GSE31207).

5.2.5 Real-Time PCR

To validate DEGs, the same RNA used for microarray was used for real-time quantitative PCR. Real-time analysis was following previously published procedures (Xiao et al., 2011). Briefly, the RNA was first treated with TURBO DNA-free DNase (Applied Biosystem, Foster City, CA) to remove trace amounts of genomic DNA. Reverse transcription was performed using Multiscribe reverse transcriptase and random primers as recommended by the manufacturer (Applied Biosystem, Foster City, CA). The fold changes between groups were evaluated using relative quantization (delta Ct method) with β -actin endogenous controls (demonstrating low variation from microarray analysis). The selection of genes was based on the strength of the findings and relevance to neurological function.

5.2.6 Behavioral assays

Behavioral assessments were performed on a separate cohort of mice 2-4 months post infection, at the time when chronic infection has developed (139). *Toxoplasma* has been

shown to impair the normal estrous cycle and makes female mice infertile at as early as 1 month post infection (188). Thus, by the time of our behavioral testing, female mice were unlikely to be cycling.

Behavioral tests were conducted in the following order: aversion to bobcat odor (a commonly used model for *Toxoplasma*-specific manipulation), pre-pulse inhibition of the acoustic startle (a predicted behavioral change from gene expression profiles in female mice to measure sensorimotor gating), open-field test (a commonly used model to assess novelty-induced activity in *Toxoplasma* infected mice), the buried food test (check olfactory ability to make sure there are no olfactory deficits would interfere with performance and produce false positive results), and social transmission of food preference test (a predicted behavioral change from gene expression profiles in male mice to evaluate olfactory based learning and memory). The strain of mice and infection conditions were the same as described earlier.

5.2.6.1 Aversion to bobcat urine

Aversion to cat urine was tested using a 3-chambered apparatus (40 x 20 x 20 cm each chamber) typically used to test social interaction (189). Mice were allowed to freely explore the chamber for 5 min each of 3 days prior to testing. Testing was performed with 50-mL vial caps containing either 1-mL of undiluted mink or bobcat urine (Murray's Lures and Trapping; Walker, WV). The goal was to test whether *Toxoplasma* manipulates the behavior of its intermediate host to increase transmission to a feline predator (bobcat) as compared to a non-feline predator (mink). Two caps with the same

odor were placed in either side chamber, keeping the middle empty. Mice were placed in the middle chamber and allowed to freely explore all chambers for 60 min. Time spent in either side chamber was automatically recorded (LabVIEW 8.5.1, National Instruments). Occupancy was calculated as the percent time spent in each odor chamber during the 60-min trial.

5.2.6.2 Sensorimotor gating

Prepulse inhibition (PPI) of the acoustic startle was used to assess sensorimotor gating as described previously (134). Briefly, the PPI test is initiated with a 5-minute acclimation period to startle chambers (San Diego Instruments, Inc., San Diego, CA), followed by different trial types: acoustic startle pulse alone (white noise, 120-decibel (dB)/100-msec); five pre-pulse trials (P1 to P5) set at 74, 78, 82, 86, or 90 dB precede the pulse by 80-msec, and finally omission of stimuli was presented to measure the baseline movement. The background noise was a white noise of 70 dB. Startle reactivity and PPI were evaluated by comparing absolute values of the startle amplitudes for different types of trials between groups.

5.2.6.3 Novelty-induced activity

Novelty-induced activity was examined in computerized Digiscan (Omnitech Electronics) activity monitors. The experimental chambers consisted of clear Plexiglas cages measuring 16×16×12 in. (40×40×30.5 cm) with a row of infrared monitoring sensors mounted every 5 cm along the perimeter at the base and a second row of sensors mounted at a height of 10 cm. The total number of beams broken was automatically

recorded over a 30-min period. For each sex, total beams broken by each infected mouse were normalized to the mean of total beams broken by the sex matched control group. For example, a normalized value for infected female mouse #1 was calculated as follows: (total beams broken by mouse #1/ average of beams broken by all control female mice) * 100%. The normalized data for infected mice were used for statistical analysis.

5.2.6.4 Olfactory Acuity

Olfaction was assessed using the buried food test (190). Mice were food deprived to 74-91% of their body weight. Testing was performed in a clean cage (11 in X 6.5 in X 5 in). Briefly, a familiarized food pellet was buried 2 cm below bedding on one side of the cage. Mice were placed in the cage as far from the food as possible. The time for the mouse to locate the food pellet was recorded using a stopwatch.

5.2.6.5 Social transmission of food preference (STFP)

Olfactory memory was assessed as described by Wrenn et al., (2004) (191), with modification to the testing apparatus as described below. The test consisted of 4 phases conducted over 3 days: habituation to food jars (Day 1), feeding demonstrator mice, and observing interaction between demonstrator and observer mice (Day 2), and testing food preference in observer mice (Day 3).

5.2.6.5.1 Jar and Food Preparation

Glass canning jars (4 oz, 6.5 cm diameter, 5.5 cm height) with a hole (1-1.4 cm diameter) cut in the centre of each lid were used to minimize spillage of food. Jars were filled 4 cm

with fresh bedding and topped with powdered food. Powdered food was created by crushing familiarized food pellets. Flavoured food was prepared by mixing powdered food with either cocoa (2%) or cinnamon (1%). Weight of the food jars were taken before and after each test to calculate the amount of food eaten.

5.2.6.5.2 Habituation to food jars

Mice were food deprived for 18 hours. One mouse from each home cage was chosen at random to be the demonstrator mouse. The demonstrator was single housed immediately prior to habituation. Two food jars containing unflavored powdered food were placed at opposite ends of the demonstrator mouse cage. Mice were allowed to freely eat for 1 hour. Food jars were then placed in the home cage for 1 hour and mice were allowed to freely eat. Pellet food was returned to all cages at the end of habituation. Demonstrator mice remained single housed and kept in an area of the housing room separate from the home cage.

5.2.6.5.3 Feeding demonstrator mice

Demonstrator mice were food deprived for 18 hours. Two jars containing the same flavoured powdered food was added to opposite ends of the cage and mice allowed to freely eat for > 1 hour. The flavoured food each demonstrator received was randomized. Demonstrators that ate > 0.2 g of flavoured food were used for the next phase.

5.2.6.5.4 Observing interaction between demonstrator and observer mice

After demonstrators were fed with flavoured food, they were returned to the home cage. Muzzle-muzzle interaction between the demonstrator and each observer mouse was recorded for 30 minutes.

5.2.6.5.5 Food preference of observer mice

Observers were food deprived for 18 hours and single housed immediately prior to testing. One food jar of each flavour was placed in each testing cage. The placement of each flavoured food jar was randomized. Mice were allowed to freely eat for 1 hour and the eating amount of cued versus amount of noncued food were recorded.

5.2.7 Statistical analysis

Data were analyzed independently for males and females using mixed-design ANOVA by SigmaStat version 3.10 (Systat Software, Inc., Chicato, IL, USA). Significant effects were explored further with lower levels ANOVAs and/or post hoc comparisons. $P < 0.05$ was used for the significance level.

5.3 Results

5.3.1 Differential effects of *Toxoplasma* on gene transcription of male and female mice

5.3.1.1 Gene expression profiling in frontal cortex in the initial cohort

A summary of the effects of *Toxoplasma* infection on gene expression of frontal cortex is depicted in Table 5.1. In infected female mice, a total of 124 gene transcripts were modulated by latent toxoplasmosis representing approximately 0.52% of the total gene set analyzed by the array. Among them, 64 (51.6%) were upregulated and 60 (48.4%) were downregulated. Similarly, a total of 153 gene transcripts representing approximately 0.64% of the total gene set were modulated in infected male mice, 80 (52.3%) being upregulated and 73 (47.7%) downregulated.

Table 5.1 Number of differentially expressed RefSeq genes and altered canonical pathways

Sex	No. of Ref Seq genes *		No. of altered pathways **
	Upregulated	Downregulated	
Male	80	73	7
Female	64	60	14

* denotes FDR-adjusted $p < 0.02$; > 1.7 fold change in expression

** denotes $p < 0.05$

There were only 5 gene transcripts that overlapped between infected female and male mice groups (*Prg2*, *Slpi*, *Sema7a*, *Amotl2*, *Vapb*). Three of these genes (*Prg2*, *Slpi*, *Vapb*) were modulated in the same direction, while two of them (*Sema7a*, *Amotl2*) in the opposite direction. There were two gene transcripts (*Tsc22d3*, *Enox2*) modulated in chromosome X in female mice, *Tsc22d3* upregulated and *Enox2* downregulated. In contrast, *Zfp185* was the only gene transcript that upregulated in chromosome X in male

mice. There were no Y chromosome genes which were modulated in male mice.

5.3.1.2 Canonical pathways among DEGs during *Toxoplasma* infection

We performed IPA on each dataset of DEGs to identify altered canonical pathways. In infected female mice, the most significant pathways ($p < 0.01$) were all signaling pathways. These included three immune response pathways (OX40 signaling pathway, allograft rejection signaling, and cytotoxic T lymphocyte-mediated apoptosis of target cells) and one disease-specific pathway (neuroprotective role of THOP1 in Alzheimer's disease). These signaling pathways appeared to be inhibited, since the majority of DEGs within these pathways were down-regulated. In infected male mice, lipid metabolic pathway “androgen and estrogen metabolism” was highly affected ($p < 0.01$).

5.3.1.3 Identification of brain-related biological processes

In both sexes, *Toxoplasma* infection resulted in remarkable changes in the expression of genes associated with the nervous system.

5.3.1.3.1 Nervous system development

In *Toxoplasma* infected female mice, a total of 15 GO categories, including 9 DEGs corresponded to nervous system development were enriched (Fig. 5.1A; *Eya1*, *Fabp7*, *Chl1*, *Sox2*, *Sema7a*, *Smarcd1*, *Nodal*, *Tnfrsf12a*, and *Tacc3*). A large proportion of these 9 genes (4 out of 9 or 44.4%; *Fabp7*, *Sox2*, *Nodal*, and *Tacc3*) corresponded to genes implicated in the brain development. More specifically, it was remarkable that 75% of the genes (3 out of 4; *Fabp7*, *Sox2*, and *Tacc3*) were implicated in forebrain

development. Further analyses revealed that dysregulation was implicated in diencephalon development, interkinetic nuclear migration, adenohypophysis development, and cell proliferation in forebrain. Also noteworthy, a good proportion of these 9 genes (3 out of 9 or 33.3%) corresponded to genes implicated in neurogenesis (*Fabp7*, *Sox2*, and *Tacc3*) as well as regulation of neuron differentiation (*Eya1*, *Sox2*, and *Tnfrsf12a*).

In *Toxoplasma* infected male mice, a total of 8 GO categories, including 9 genes corresponding to nervous system development were enriched (Fig. 5.1B; *Foxn4*, *Dpysl2*, *Arsb*, *Sema7a*, *Bmp6*, *Ablim1*, *Slit1*, *Aqp1*, and *Glis2*). Most altered processes involved the single gene of *Slit1*, e.g. telencephalon cell migration, axon extension involved in axon guidance, tangential migration from the subventricular zone to the olfactory bulb, and regulation of axon extension involved in axon guidance. Similarly, ventricular system development and lateral ventricular development involved another common altered gene: *Aqp1*.

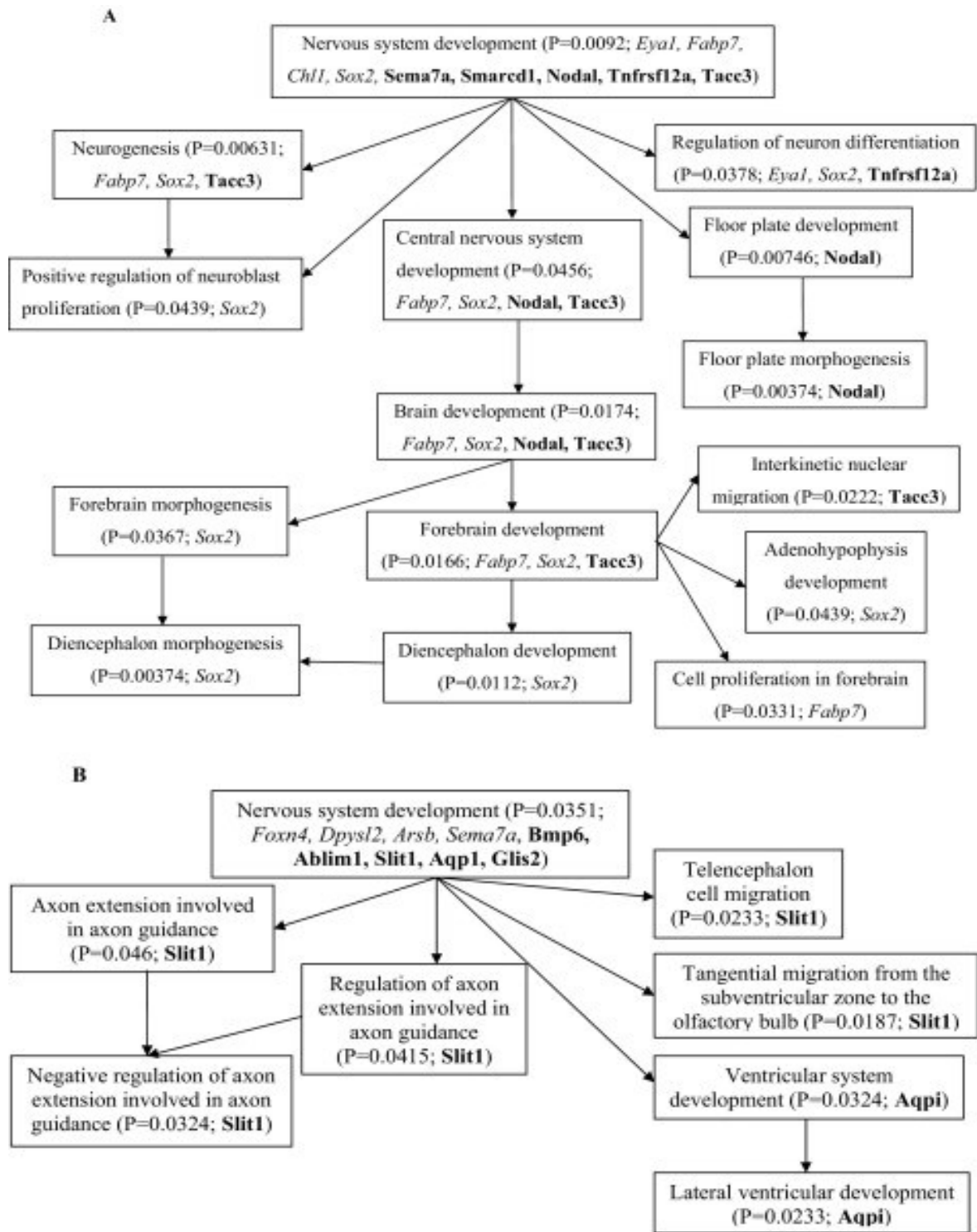


Fig 5.1 Enriched biological processes and their relationships involved in nervous system development by *Toxoplasma* infection.

A) Female mice, **B)** Male mice. Shown are the biological processes with significant p-value changes in mice altered by persistent infection. Altered genes in each process are listed. Upregulated genes appear in bold, and downregulated genes are in italic.

5.3.1.3.2 Neurological system process

In *Toxoplasma* infected female mice, a total of 8 GO categories, including 12 genes exhibit signs of enrichment in neurological system process (Fig. 5.2A; *Fabp7*, *Chl1*, *Myc*, *Sox2*, *Ppef2*, *Rdh8*, *Pdyn*, *Olfr10*, *Olfr1404*, *Olfr142*, *Olfr156*, and *Olfr622*), with the majority being upregulated. Interestingly, there were 5 olfactory receptors, all of which were upregulated. However, the neuromuscular process, including equilibrioception and pre-pulse inhibition appears to be inhibited, since all the DEGs (*Fabp7*, *Sox2*) within this process were down-regulated. Similarly, the sensory perception of sound could be inhibited also because genes within this process (*Myc*, *Sox2*) were downregulated.

In *Toxoplasma* infected male mice, a total of 6 GO categories, including 14 genes exhibit signs of enrichment in neurological system process, with the majority being down-regulated (Fig. 5. 2B; *Drd4*, *Olfr670*, *Olfr373*, *Olfr213*, *Olfr485*, *Olfr207*, *Olfr1496*, *Olfr493*, *Olfr47*, *Tas2r140*, *Kcnv2*, *Aqp1*, *Olfr1436*, and *Olfr564*). It is interesting to note that 92% of these genes (13 out of 14) corresponded to genes implicated in cognition as well as sensory perception. More specifically, 76.9% genes (10 out of 13) were implicated in sensory perception of smell. Among them, 8 were downregulated (*Olfr670*, *Olfr373*, *Olfr213*, *Olfr485*, *Olfr207*, *Olfr1496*, *Olfr493*, *Olfr47*) and 2 were upregulated (*Olfr1436*, *Olfr564*).

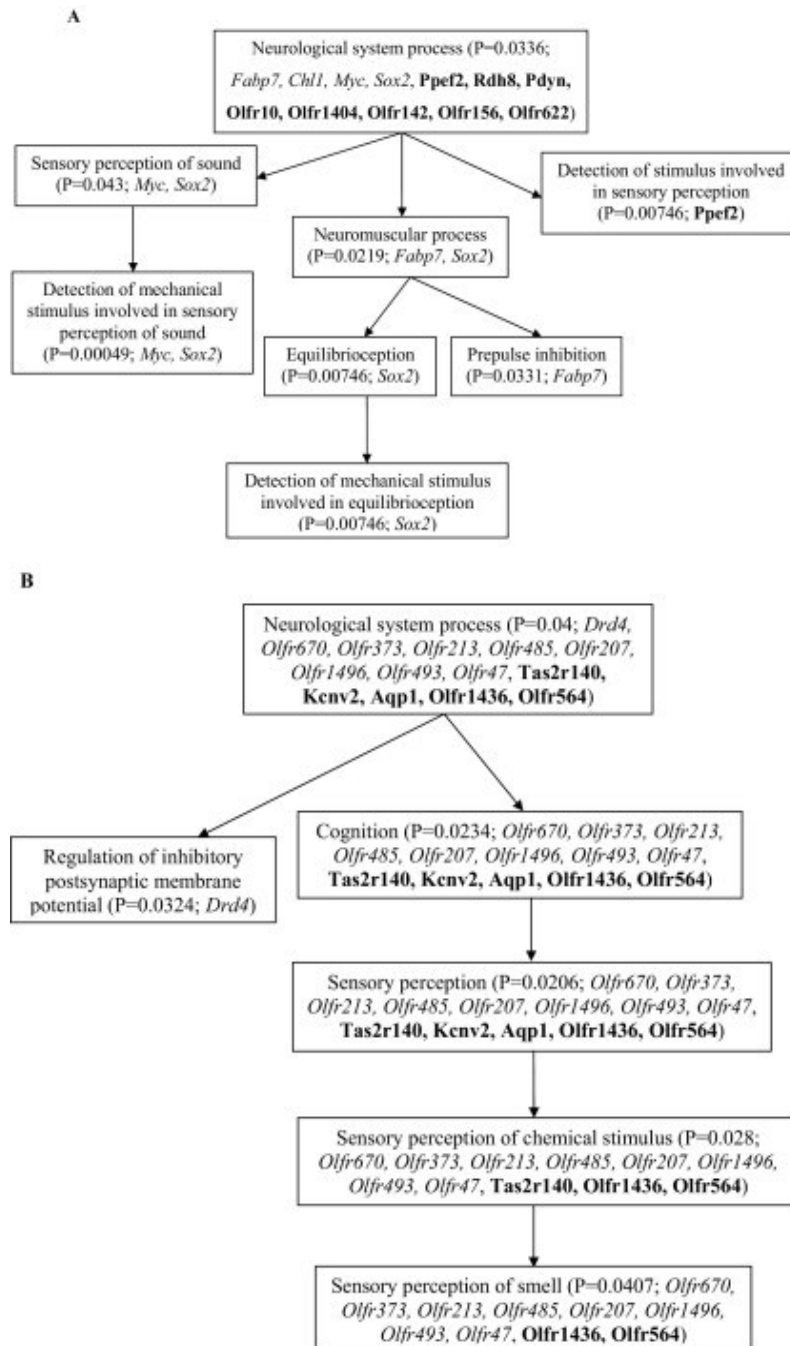


Fig 5.2 Enriched biological processes and their relationships involved in neurological system process by *Toxoplasma* infection

A) Female mice, B) male mice.

Shown are the biological processes with significant p-value changes in mice altered by persistent infection. Altered genes in each process are listed. Upregulated genes appear in bold, and downregulated genes are in italic.

5.3.1.4 Real time PCR validation

In *Toxoplasma* infected female samples, we analyzed one up-regulated gene (*Sema7a*) and three down-regulated genes (*Enox2*, *Eyal*, *Pabp7*, *Prg2*), and in infected male

samples, four up-regulated genes (*Bmp6*, *Lmf2*, *Slit1*, *Olfrl436*) and three down-regulated genes (*Jph2*, *Prg2*, *Drd4*). In general, the fold-changes obtained by qPCR were lower than those obtained in the microarray analyses. No signal was detected in qPCR for *Olfrl436* by 45 cycles. Out of the 11 genes tested, 10 showed a positive correlation with the microarray expression data ($r = 0.654$) (Fig. 5.3).

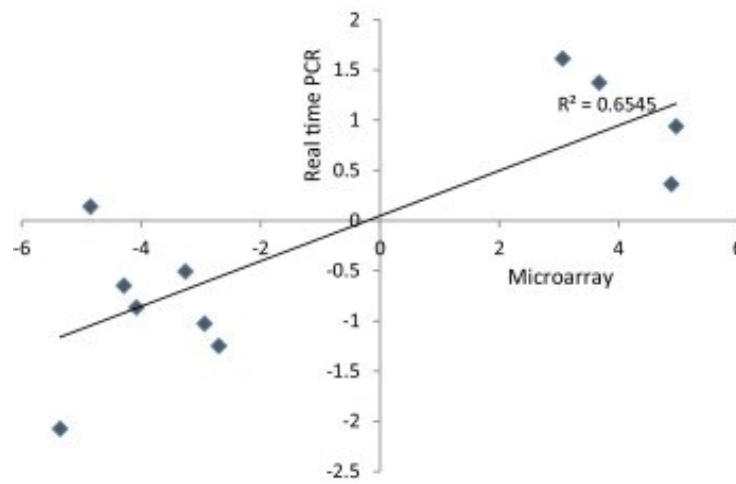


Fig 5.3 Correlation between microarray and real-time PCR data

The scatter plot compares mean expression of infected cells/ controls ratios for nine genes. Each point represents the infected cells/ controls ratio from the microarray (X-axis) and real-time PCR (Y-axis). The Pearson correlation coefficient is indicated on the scatter plot. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of the published version of this chapter (140).

5.3.1.5 Comparison analysis between infected female and male mice

To identify differences in neurological functioning between infected females and males, the genes that were differentially regulated were compared using IPA. The analysis

revealed that behavior function was affected more in infected females than males by comparing the extent to which the significances change ($7.67\text{E-}3$ vs $4.49\text{E-}2$). Infected females had changes in genes involved in 5 specific behaviors: place aversion, ultradian rhythm, feeding, acoustic startle response, and mechanical allodynia, while infected males had changes in genes involved in only 1 specific behavior: olfactory memory. Of this list of behaviors, olfactory memory and pre-pulse inhibition were tested further, due to the association of these behaviors with schizophrenia.

5.3.2 Differential effects of *Toxoplasma* on the behavior of male and female mice

5.3.2.1 Effect of *Toxoplasma* infection on body weight and antibody titers of male and female mice

Both male and female mice infected with *T. gondii* showed a significant decrease in body weight (Table 5.2). This decrease was observed 4 weeks post infection, prior to the start of all behavioral tests, and remained throughout the course of experiments. High anti-*Toxoplasma* antibody titres were found in sera of infected male (3.82 ± 0.06) and female (3.85 ± 0.05) mice. There were no differences in serum antibody titers between the infected male and female group ($p = 0.10$).

Table 5.2 Average body weight (g) of infected and control mice throughout the course of behavioral test

	Male		Female	
	Control	Infected	Control	Infected
Preinfection	26.8 ± 0.5	26.6 ± 0.5	21.3 ± 0.5	20.8 ± 0.5
1 mpi	28.3 ± 0.6	24.7 ± 0.6	23.5 ± 0.7	18.7 ± 0.7
2 mpi	29.2 ± 0.6	27.1 ± 0.5	24.8 ± 0.8	19.5 ± 0.9
3 mpi	31.2 ± 0.6	29.0 ± 0.5	26.9 ± 1.1	20.1 ± 1.1
4 mpi	32.6 ± 0.6	31.4 ± 0.7	26.6 ± 0.9	22.9 ± 1.4

All data are mean values ± SEM (g).

mpi denotes month post infection

5.3.2.2 Differential effects of *Toxoplasma* on the behavior of male and female mice

We found that infection produced differential effects on avoidance of feline odor, olfactory memory, and locomotor activity in male and female mice. We first tested whether the *T. gondii* specific manipulation of decreasing rodent aversion to cat odor is seen in both sexes. Two-way ANOVA for the data for female mice found a significant effect of odor, $F(1, 35) = 4.496$, $P = 0.042$. Planned t-test detected that infected female mice spent significantly more time near bobcat odor as compared with time spent near mink odor ($t = 3.11$, $Df = 32$, $P < 0.05$). In contrast, no differences in time spent near either odor between infected and uninfected male mice were observed, all $F < 1$; $P_s > 0.05$ (Fig. 5.4).

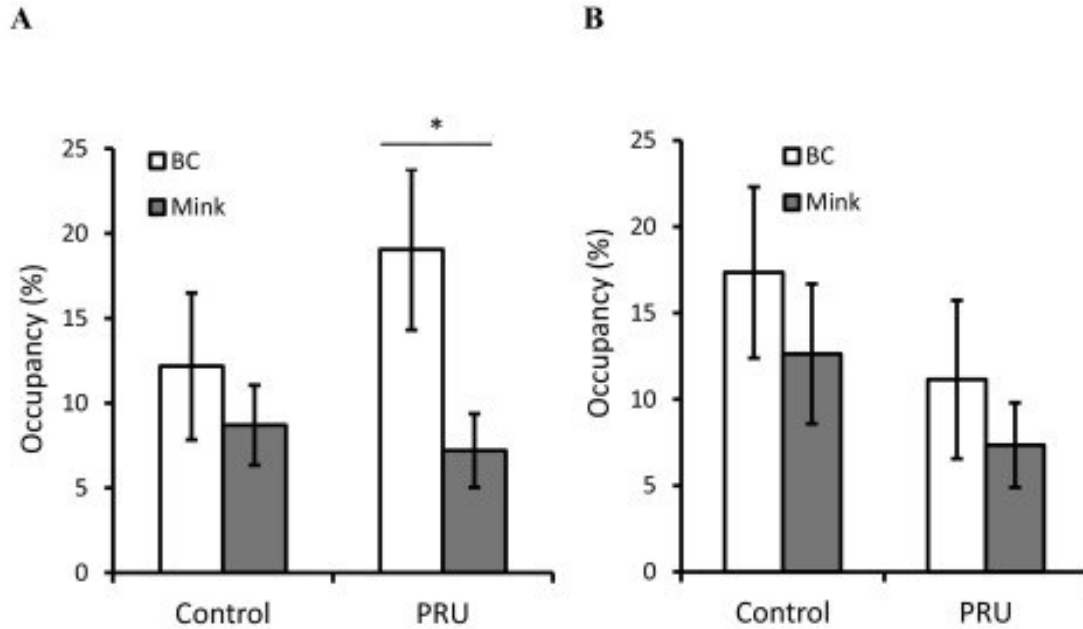


Fig 5.4 Effect of infection on aversion to cat odors during a 60-min trial. Ordinates depict the occupancy in the target quadrant

A) Infection abolished aversion to bobcat urine in female mice. * denotes $p < 0.05$ relative to mink odor. Female control mice, $n=10$; infected female mice, $n=8$

B) Infection did not affect aversion to cat odors in male mice. Male control mice, $n=10$; infected male mice, $n=15$. Error bars denote SEM.

We tested olfactory memory through the social transmission of food preference (STFP) task. No effect of food novelty was seen in the female group, $F(1, 25) = 1.245$, $P = 0.277$. In contrast, two-way ANOVA detected a significant effect of food novelty for male mice, $F(1, 31) = 9.502$, $P = 0.005$. Planned t-test showed that control males

consumed significantly less novel food than familiar food ($t = 4.29$, $Df = 28$, $P < 0.05$).

No difference in novel and familiar food consumed was found in infected male mice, $P > 0.05$ (Fig 5.5).

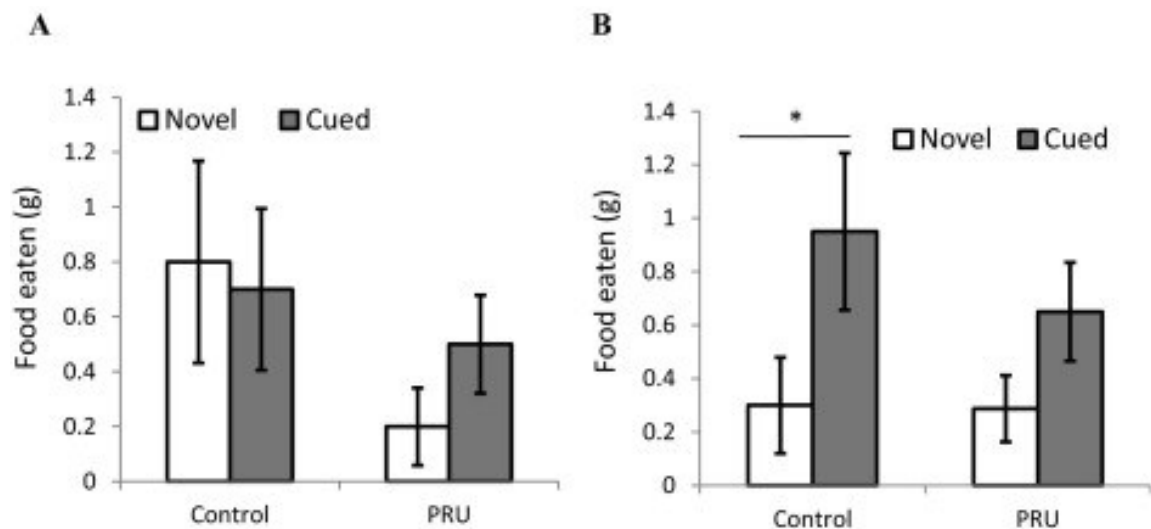


Fig 5.5 Effect of infection on social transmission of food preference (STFP).

Ordinate depicts preference for the food

A) No infection effects were found in STFP in the female group. Female control mice, $n=8$; infected female mice, $n=5$.

B) infection caused a deficit in STFP in the male group. Male control but not infected male mice had significant preference for the cued food; * denotes $p < 0.05$. Male control mice, $n=8$; infected male mice, $n=8$. Error bars denote SEM.

To determine whether differences seen in STFP were because of differences in the number of interactions during the observation phase or impairment in smelling ability, we assessed social interactions and tested olfactory acuity via the buried food task. We found no difference in the number of observer mouse sniffs between control and infected females ($F(1, 12) = 0.429, P = 0.526$) or males ($F(1, 15) = 1.042, P = 0.325$). In addition, we observed no significant effects of infection on olfaction in males and females as evidenced by no differences between the groups in time spent finding the buried food pellet, $F(1, 39) = 0.418, P = 0.522$.

We measured the effect of *Toxoplasma* infection on novelty induced activity in male and female mice using open field test. As depicted in figure 5.6, infection had the opposite effects on male and female mice. When the distance traveled by infected mice was normalized to the one in the sex-matched control group, one-way ANOVA detected that infected female mice were hyperactive while infected male mice displayed hypoactivity, $F(1, 21) = 8.07, P = 0.01$.

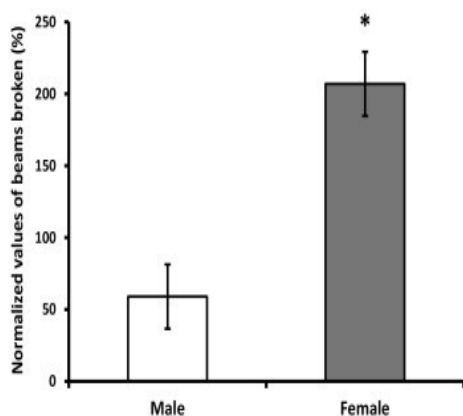


Fig 5.6 Female infection increased locomotor activity in the open-field arena compared with male

The ordinate depicts the normalized values of beams broken by the infected mice. For each sex

group, total beams broken by each infected mouse were normalized to the mean of total

beams broken by the corresponding control group. Female control mice, n=10; infection female mice, n=8; male control mice, n=10; infected male mice, n=14. * denotes $p = 0.015$ relative to male mice. Error bars denote SEM.

No effects of infection were found on the magnitude of the acoustic startle or pre-pulse inhibition in either sex (Fig. 5.7). Two-way repeated measures ANOVA for the data did not reveal any effect of infection, all $P_s > 0.05$.

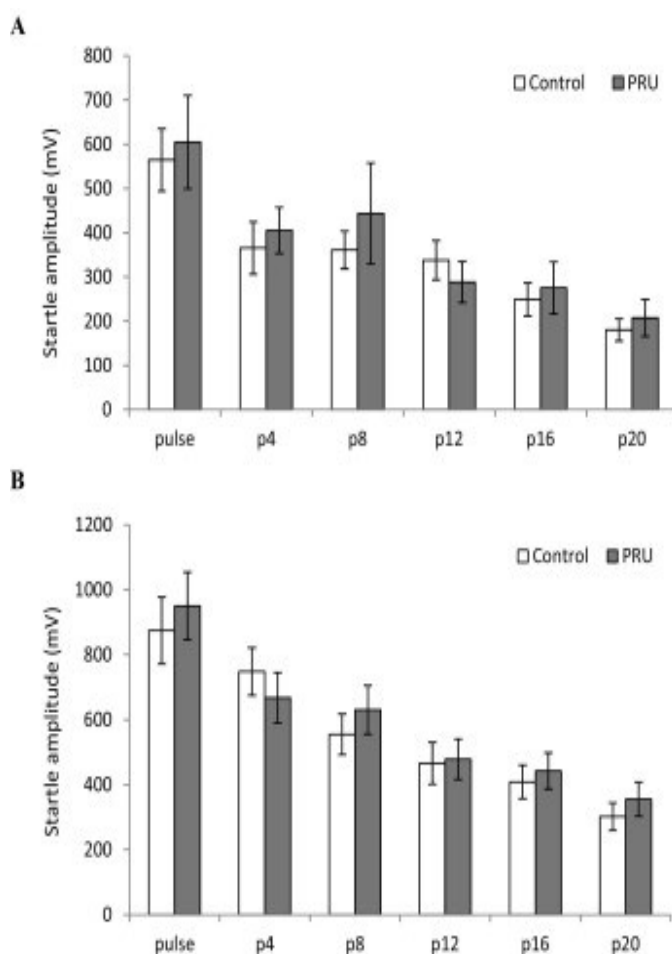


Fig 5.7 Infection did not influence prepulse inhibition in A) females and B) males

X-axis: p4, p8, p12, p15, and p20 are intensities of different prepulse trials above the background noise level (70 dB). Error bars denote SEM. Female control mice, n=10; infected female mice, n=8; male control mice, n=10; infected male mice, n=14;

5.4 Discussion

A convincing body of evidence exists to support that *Toxoplasma* can cause specific behavioral changes in its host. In this study, we sought to identify the molecular changes that result from persistent *Toxoplasma* infection in mice. Using a whole genome microarray, we observed that male and female mice exhibited unique frontal cortex transcriptome responses to infection. The sex-dependent changes in gene expression were supported by or agreed with sex-specific effects of *Toxoplasma* infection on several behavioral abnormalities. Consistency between differences in gene expression and behavior strengthens the significance of sex-dependent effects of *Toxoplasma*, although additional studies are required to more directly elucidate gene expression-behavior relations. The study suggests that specific sex-parasite interactions influence the behavior of intermediate hosts in important ways and that these behavioral changes increase the likelihood of predation.

5.4.1 Neurological effects in male mice

In male mice, the most striking alteration in expression was found for the genes involved in cognitive perception of smell with a very selective tropism to olfactory receptors (Fig. 5.2B). The majority of altered olfactory receptors appeared to be down-regulated (8 out of 10), although there were 5 different olfactory receptors up-regulated in female mice. It is possible that some odor molecules that act as ligands for repressed receptors have been eliminated, while other molecules that specialize in binding to the increased receptors have been added. Slit1 (slit homolog 1), significantly upregulated, was involved in several altered processes of the central nervous system in male mice. Slit1 expressed in

the septum appears to repel olfactory bulb axons as they project into the lateral olfactory tract (192). Consequently, the increased expression of Slit1 could inhibit the processing of olfactory information. These changes are specific and suggest the existence of subtle dysregulation of brain function because olfactory acuity is still intact. This is consistent with a recent report that *Toxoplasma* manipulates the rodent's behavior mainly through a specific compromise in olfaction without affecting other behaviors (138). Although the olfactory changes could increase the risk of specific predation since this behavior is a primary mean of gathering information for a nocturnal species with poor visual acuity, it should be noted that the infected male mice did not show preference for bobcat urine odor. An explanation for this outcome is that these altered olfactory genes might not be responsible for cat odor detection. This hypothesis seems to be consistent with our finding that the same genes were not affected in infected female mice that did exhibit cat odor attraction, possibly as a result of the parasite manipulation of the olfaction mechanisms. In fact, given the deficient performance of infected male mice in the test for social transmission of food preference, it is tempting to speculate that the olfaction genes affected in male mice might be involved in cognitive processing of olfactory stimuli.

Another possible reason for deficient memory observed in infected male mice in the test could include the altered expression of DAD4 (dopamine receptor D4). Laviolette et al. (2005) demonstrated that blockade of DAD4 directly in the medial prefrontal cortex (mPFC) blocked the acquisition of olfactory fear conditioning in awake, behaving animals as well as the encoding and expression of emotional learning in individual

neurons of the mPFC (193). They inferred that signaling through mPFC DAD4 substrates may be involved in the regulation of neuronal plasticity mechanisms and memory processes necessary for adaptive emotional learning and sensory processing. It is possible that the downregulated DRD4 observed in male mice (Fig. 5.2B) contributes to the deficits in the STFP (Fig. 5.5). Indeed, the dopaminergic system has been implicated in social learning (194). Moreover, the deficit is in agreement with previous reports which found that rodents infected by *Toxoplasma* become less neophobic and more willing to approach a novel object or odor (144). In contrast to male mice, food preference was not observed in the female mice in the current study. This observation is inconsistent with some previous reports (194, 195). However, Ryan et al.'s reported (2010) a similar pattern with no preference for the cued food in female C58/J and C57BL/6J mice as measured by the amount of food consumed (196). This may be due to the difference in the mouse strains used for the experiments. Further studies are needed to elucidate the difference for this test.

Abnormalities in dopamine signaling have been linked to schizophrenia (197). The atypical antipsychotic clozapine, which is effective in treating refractory schizophrenics, displays a 10-fold higher affinity for the D4 receptor than the D2 and D3 receptors (198, 199). These facts suggest that the D4 receptor is a potential target for mediating clozapine's antipsychotic effects. Previously, we have shown a possible association between *Toxoplasma* infection and an increased risk of human neuropsychiatric disorders (174). In addition, there is evidence that polymorphisms present in the DAD4 gene play a role in the etiology of autism (200). Recent studies suggest *Toxoplasma* infection may

trigger development of autistic spectrum disorders (201). It is of note that increased levels of dopamine have been observed in the brains of mice chronically infected with *Toxoplasma* (20, 150).

5.4.2 Neurological effects in female mice

In female mice, *Toxoplasma* infection appears to modify multiple neurological processes related to maturation of the forebrain, neurogenesis, sensory and motor coordination (Fig. 5.1A, 5.2A). Several studies have investigated the innate fear of laboratory rodents toward cat odors (202-204). These studies have delineated a neuroanatomical circuit in the medial hypothalamic zone (MHZ), with input from several forebrain sites as critically involved in the organization of innate defensive behavior. Interestingly, the specific alteration in expression of genes involved in maturation of the forebrain (diencephalon and adenohypophysis included) that correlated with decreased aversion to cat odor (Fig. 5.4) was observed in infected female mice. Moreover, the specific modulation of the development of forebrain was observed previously on neural cells infected by *Toxoplasma* (185). These alterations appeared specific to only those behavioral traits likely to enhance predation toward definitive host is in agreement with previous reports (169) and supports the manipulation hypothesis.

An effect of *Toxoplasma* infection on neuromuscular processes, including equilibrioception and the acoustic startle was indicated by gene expression in female mice (Fig. 5.2A). No changes in the acoustic startle responses were found although prior studies have reported motor deficits (such as motor function, balance, grip strength and

coordination) in infected mice (139, 173). The reasons for our inability to demonstrate differences in the acoustic startle are not clear. There is a possibility that the test used were not sensitive enough to detect neuromuscular deficits should these deficits actually exist in infected mice. In addition, the effect of latent toxoplasmosis on locomotor activity showed an opposite direction in infected male and female mice (Fig. 5.6). The finding is consistent with previous results which reported that decreased activity in infected male (173), but increased activity in female (134).

The data presented here provide evidence that *Toxoplasma* can produce sex-dependent changes in mice. Our results are consistent with previous findings on sex differences in human behaviors induced by latent toxoplasmosis (15, 18) and in susceptibility to *Toxoplasma* infection in mice (145). Potential explanations for this phenomenon might include: a) differences in the localization of bradyzoites to the brains of male and female rodents, with differential functional consequences; b) sex difference in the length of cyst burden; c) differential sex-related neuroimmunological responses to *Toxoplasma* infections (205); and d) the general increased susceptibility of female mice over male mice to *Toxoplasma* infection could be playing a role in the observed differences.

5.5 Conclusion

Overall, this chapter demonstrates sex-dependent effects of *Toxoplasma* on gene expression and behaviors in its intermediate host. Our results support the notion that *Toxoplasma* has a high degree of selection for host manipulation. The postulated

mechanisms that might contribute to the development of various neurological and psychiatric symptoms deserve further investigation.

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CHAPTER 6:
**The Effect of Mouse Age during First Exposure to *Toxoplasma gondii* on
Behavior and Brain Alterations**

Abstract

Toxoplasma gondii (*T. gondii*) infection has been shown to manipulate rodent behavior. Studies have centered around the effect of first exposure during adulthood or congenitally on behavioral manipulation. Infection during adolescence, a period during which the brain is still developing and maturing, has not been studied. Here we look at the effect of *T. gondii* exposure during adolescence and adulthood, specifically targeting changes to pre-pulse inhibition of the acoustic startle behavior in mice. In addition, we describe changes in monoamines and serum antibodies to the NR2 subunit of the *N*-methyl-D-aspartate receptor (NMDAR). This study shows that the age at which mice are infected with *T. gondii* is an important factor in the development of behavior and brain alterations.

6.1 Introduction

Rodents infected with the protozoan parasite *Toxoplasma gondii* (*T. gondii*) have been shown to exhibit altered behaviors. Studies have focused on the effect of chronic *T. gondii* infection, with first exposure either congenitally or during adulthood (e.g. 6-8 weeks old) (134, 138, 141, 142, 151, 178, 206). Surprisingly, no work has been done on the effect of first exposure to *T. gondii* during adolescence (e.g. 4-5 weeks old).

The effect of prenatal exposure to pathogens on brain development and behavior has been a large focus of research (207). However, the effect of exposure to stressors during adolescence on adult behavior has been gaining attention (88, 208, 209). It is now better understood that the brain is vastly changing during adolescence, and disruption during this critical period could have significant consequences later in life. Important brain changes during adolescence include maturation of interneuron, projections of mesocortical dopamine neurons, and decreased glutamatergic synapse density (19). Therefore, *T. gondii* infection during adolescence may result in greater or different behavioral abnormalities than previously observed with adult infection due to disruption of on-going brain maturation. In order to test this hypothesis, we directly compared the brain and behavioral effects of adolescent (juvenile) and adult *T. gondii* exposure in mice.

6.2 Material and Methods

6.2.1 Animals

Male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animal protocols were reviewed and approved by the Animal Care and Use Committee of Johns Hopkins University (JHU). Mice were housed 5 per cage with 14.5/ 9.5 hours of light/dark cycle with free access to food and water.

6.2.2 *Toxoplasma* tachyzoite purification

Prugniald (PRU) tachyzoites were maintained in human foreskin fibroblast monolayers (HFF) and purified as previously described (CITE). Mice were either intraperitoneally mock-infected with sterile Dulbecco's phosphate buffered saline (DPBS) or infected with 400 tachyzoites (2 parasites/ μ L) at 4.5 or 9 weeks of age.

6.2.3 Behavioral Testing

Behavioral testing was performed 2-4 months post-infection (mpi), with the following tests and order used: open field, pre-pulse inhibition of the acoustic startle (PPI) with saline administration; PPI with MK801 (0.1 and 0.3 mg/kg) administration; PPI with saline administration; PPI with amphetamine (5 mg/kg and 10 mg/kg) administration. Mice were given 1 week of rest between each test. All testing was conducted by a female experimenter.

6.2.3.1 Novelty-induced activity of the open field

Novelty-induced activity was assessed in the open field (San Diego Instruments).

Individual mice were placed in the center of the arena and allowed to freely move.

Central, peripheral, and rearing beam breaks were automatically recorded in 5 minute intervals over the 30 minute trial. The periphery was designated as 2.5 cm on all sides.

Total activity was calculated by summing peripheral and central beam breaks.

6.2.3.2 Pre-pulse Inhibition of the Acoustic Startle

Sensorimotor gating was assessed using pre-pulse inhibition of the acoustic startle (PPI).

Mice were intraperitoneally injected with 200 μ L bacteriostatic 0.9% sodium chloride (Saline) (Hospira, INC; Lake Forest, IL), 0.1 mg/kg or 0.3 mg/kg (+)-MK801 hydrogen maleate (Sigma-Aldrich), and D-amphetamine hemisulfate salt C-II (Sigma-Aldrich) 5 min prior to placement in PPI chambers (San Diego Instruments). All drugs were diluted with saline and prepared fresh in the morning of use for each individual mouse. In the testing apparatus (San Diego Instruments), mice were acclimatized to a 76dB background noise for 5 min. They were then given 10 presentations of a 120dB pulse and 10 presentations of no pulse. This was followed by 5 presentations in randomized order of a 120 dB pulse, no pulse, or pre-pulses with a pulse (77, 78, 80, 84, 88 dB- 120 dB). The intervals between each presentation varied from 10-19 seconds. Mean PPI % was calculated by averaging all PPI% values for presentations of all pre-pulses for each experimental group. The mean PPI% for each infection group was normalized to that of age-matched infection controls, and the ratio multiplied by 100 to convert to percent.

6.2.4 Brain Processing for H&E Staining

Brains were fixed using standard procedures. Briefly, mice were anesthetized and blood cleared with 1X phosphate-buffered saline (PBS). Brains were perfused with 4% paraformaldehyde (PFA) and once removed from skull, immersed in 4% PFA overnight at 4°C. The following day PFA was removed and brains immersed in 10% sucrose dissolved in PBS (3X) overnight at 4°C. On the next day, the sucrose solution was replaced with 30% sucrose solution in 3X PBS and stored at 4°C until the brains sank. At this point, brains were quickly frozen in -50°C methyl-butane and stored at -80°C until sectioning.

Hemispheres were separated using a razor blade. One hemisphere was sectioned using a sliding microtome (Fisher Scientific, HM450). Each section (40 µM) was immediately placed in anti-freeze buffer (pH 6.5) and stored at -20°C until staining.

Sections were stained with hematoxylin and eosin (H&E) following standard procedures. Parasite cysts were visualized and counted under brightfield illumination in a blinded manner.

6.2.5 Infectivity Confirmation

Blood was taken at the culmination of behavioral testing and centrifuged at 10,000xg to collect serum. Total anti-Toxoplasma IgG was measured via enzyme-linked immunosorbent assay (ELISA) as described previously (174).

6.2.6 NR2 Antibody Measurements

Serum antibodies to NR2 were measured using Gold Dot NR2 Antibody Test Kit (CIS Biotech, Inc; GD1-001). The kit protocol was followed with one modification. Rather than incubation with Protein A-HRP, samples were incubated with anti-mouse IgG (1:40000) and the reaction later developed with ABTS peroxidase substrate.

6.2.7 Monoamine Analysis

Monoamine measurements were made using high-performance liquid chromatography (HPLC) analysis. Brain regions obtained from each animal were weighed, ultrasonicated in 0.01 M perchloric acid, and centrifuged at 20,000 xg for 15 min. Concentrations of norepinephrine (NE), dopamine (DA), 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were measured in brain tissue extracts by HPLC with electrochemical detection as described earlier (210, 211). In brief, the analytical column was Sunfire C-18 (5 μ m, 4.6 \times 150.0 mm; Waters); the mobile phase was 0.01 M sodium dihydrogenphosphate, 0.01 M citric acid, 2 mM sodium EDTA, 1 mM sodium octylsulfate, 10% methanol, pH 3.5 at flow rate 1.0 ml/min and temperature 25°C. The installation consisted of 717 Plus automated injection system, 1525 binary pump, a thermostat (all from Waters, USA) and a Coulochem III electrochemical detector (ESA). The electrode was set at +0.75 V. Peak areas and monoamine concentrations were calculated with Breeze 3.3 software (Waters). Contents of NE, DA, DOPAC, HVA, 5-HT and 5-HIAA were calculated as ng/mg of tissue weight.

6.2.8 Statistical Analysis

Data were first checked for normality and equal variance using SPSS (v.21). If failed, non-parametric Mann-Whitney U test was performed to compare age-matched DPBS and infection groups. A $p \leq 0.05$ was considered significant.

6.3 Results

We first determined whether the age at which a mouse is initially exposed to *T. gondii* affects susceptibility to the psychostimulants MK-801 or amphetamine in pre-pulse inhibition (PPI) of the acoustic startle. As indicated by data normalized to mock-infected control mice, we found different alterations in PPI upon psychostimulant administration, dependent on the age mice were first infected with *T. gondii* (Fig 6.1). Mice infected with *T. gondii* as juveniles showed decreased PPI with 0.3 mg/kg MK-801 compared with saline and 0.1 mg/kg. In contrast, mice infected as adults displayed increased PPI with 0.3 mg/kg MK-801 as compared with saline and 0.1 mg/kg MK-801 (Fig 6.1A). Differential effects due to the age of infection were also observed with amphetamine administration (Fig 6.1B). We found that while juvenile infected mice did not show significant disruption in PPI with amphetamine, mice infected as adults had increased PPI with amphetamine administration (10 mg/kg). Regardless of age of infection, both doses of MK-801 increased startle while both doses of amphetamine decreased startle in all groups (data not shown). Taken together, our data indicate that the age at which mice are administered *T. gondii* can differentially impact susceptibility to psychostimulants, decreasing PPI in mice exposed as juveniles versus facilitating PPI in mice exposed as adults.

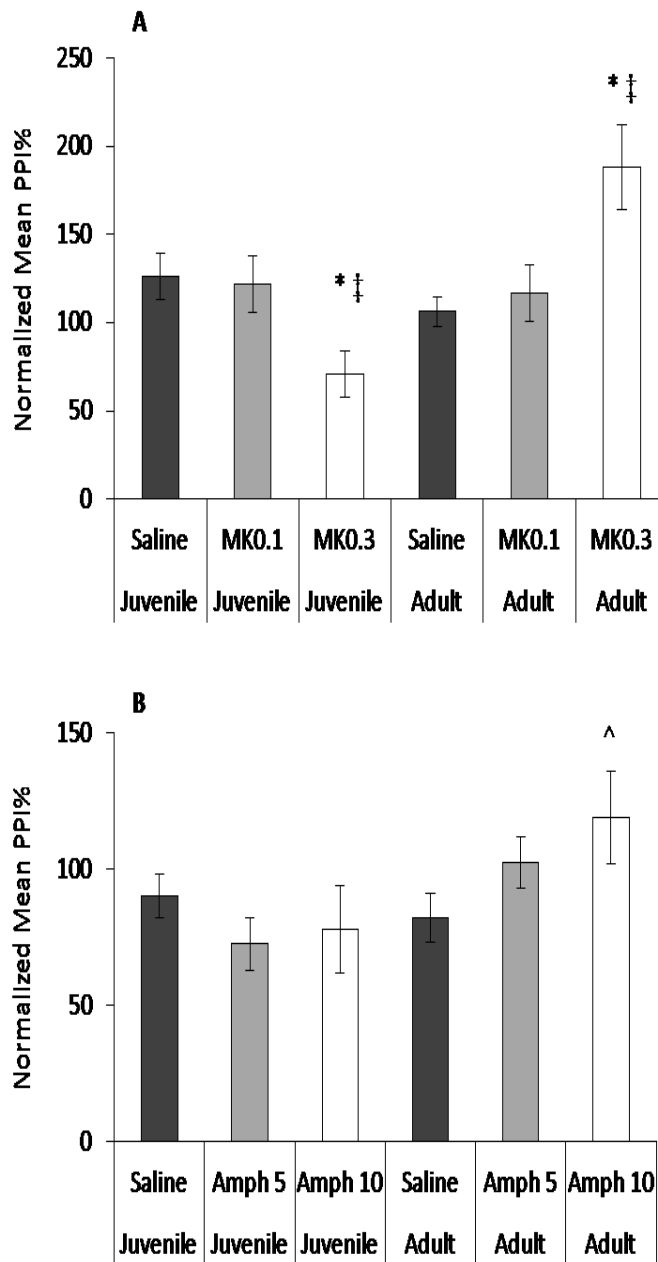


Fig 6.1 Age of host when infected with *T. gondii* affects pre-pulse inhibition (PPI) susceptibility to MK-801 and Amphetamine

A) PPI alterations with 0.1 and 0.3 mg/kg MK-801.

B) PPI alterations with 5 and 10 mg/kg amphetamine.

Juvenile saline and amphetamine, n=15; Adult saline and amphetamine, n=18

* denotes $p < 0.05$ compared to saline; ‡ denotes $p < 0.05$ compared to 0.1mg/kg; ^ denotes $p = 0.055$ compared to saline

To rule out the possibility that differences in behavior between the juvenile and adult groups are due to differences in infectivity, we measured mouse body weights, serum antibodies to *T. gondii*, and parasite cysts in the brain (Table 6.1). We found that prior to

infection (0 days post infection; dpi) and at the end of behavioral testing (117 dpi), mouse body weights did not differ between the infected and control groups for either age of infection. Yet, at the start of behavior testing (70 dpi) both juvenile and adult infected groups weighed significantly less than controls ($p < 0.05$). Infection at either age elevated serum anti-*Toxoplasma* antibodies by 117 dpi, but these levels did not differ between juveniles and adults. Furthermore, there was no significant difference in the number of parasite cysts found in the brains of juvenile and adult infected mice.

Table 6.1 Mouse weights, anti-IgG levels in serum, and Cyst numbers

	Juvenile		Adult	
	DPBS	PRU	DPBS	PRU
0 dpi (g)	18.5 ± 0.7	18.9 ± 0.5	25.8 ± 0.5	26.9 ± 0.4
70 dpi (g)	29.0 ± 0.7	26.3 ± 1.3*	30.6 ± 1.1	28.6 ± 0.6*
117 dpi (g)	30.1 ± 0.6	28.4 ± 1.5	30.3 ± 0.5	29.5 ± 0.5
IgG (A.U.)	0.04 ± 0.004	0.97 ± 0.017*	0.08 ± 0.025	0.98 ± 0.019*
Cyst (#/section)	0	1.7 ± 0.34	0	1.6 ± 0.85

* denotes $p < 0.05$ as compared with age matched control. Data represents mean ± SEM.

Body weights and serology: Juvenile DPBS, n=10; Juvenile PRU, n=15; Adult DPBS, n=10; Adult PRU, n=18

Cyst counts: Juvenile and Adult DPBS, n=3; Juvenile and Adult PRU, n=4-5. 5 sections per mouse were stained and averaged.

As differences in PPI susceptibility to amphetamine may be related to monoamine changes, we measured the levels of neurotransmitters and their metabolites in the striatum and hippocampus of mice chronically infected with *T.gondii* (Table 6.2). We found no monoamine changes in the hippocampus of mice infected as adults, as compared with DPBS infection controls. In contrast, the homovanillic acid (HVA) to dopamine (DA) ratio and 5-Hydroxyindoleacetic acid (5H1AA) to serotonin (5HT) ratio were significantly increased in the hippocampus of mice infected as juveniles, while DA, norepinephrine (NE), and 5HT were significantly decreased by infection. In the striatum, regardless of age infected, *T. gondii* lead to a decrease in DA and an increase in HVA/DA. This suggests that juvenile infection increases serotonin metabolism in the hippocampus and dopamine metabolism in the hippocampus and striatum. In contrast, adult infection may decrease DA production in the striatum. Thus, our findings indicate that the age of the host when first exposed to *T. gondii* can differentially alter neurotransmitters in the hippocampus and striatum in adulthood.

s in the striatum and hippo

		Striatum				Hippocampus			
		Juvenile		Adult		Juvenile		Adult	
		DPBS (n=10)	PRU (n=9)	DPBS (n=10)	PRU (n=10)	DPBS n=10)	PRU (n=9)	DPBS (n=10)	PRU (n=10)
Dopamine and Dopamine Metabolites									
DA (pg/mg)		12012.4 ± 334.6	* 9859.6 ± 814.8	13108.0 ± 797.3	* 9357.2 ± 789.2	44.8 ± 6.1	* 28.8 ± 5.9	43.6 ± 9.8	36.4 ± 6.9
DOPAC (pg/mg)		955.08 ± 56.3	* 774.4 ± 85.7	1178.4 ± 92.2	1178.14 ± 187.8	13.7 ± 1.9	11.1 ± 2.6	22.6 ± 4.4	14.4 ± 3.6
DOPAC/DA		0.08 ± 0.004	0.08 ± 0.005	0.09 ± 0.004	* 0.12 ± 0.013	0.34 ± 0.13	0.43 ± 0.25	0.66 ± 0.51	0.40 ± 0.13
HVA (pg/mg)		1504.0 ± 70.5	1422.4 ± 135.6	1720.5 ± 138.9	1501.4 ± 93.8	109.3 ± 7.1	127.7 ± 12.7	101.6 ± 15.9	100.36 ± 13.0
HVA/DA		0.13 ± 0.005	* 0.15 ± 0.008	0.13 ± 0.006	* 0.17 ± 0.011	2.74 ± 0.30	* 5.12 ± 0.53	2.76 ± 0.34	3.18 ± 0.35
Norepinephrine									
NE (pg/mg)		168.1 ± 15.3	139.6 ± 22.2 *	223.8 ± 14.1	* 164.5 ± 21.3	224.1 ± 12.2	* 151.6 ± 19.5	159.4 ± 16.3	193.5 ± 10.2
Serotonin and Serotonergic Metabolites									
5HT (pg/mg)		888.8 ± 79.0	1043.1 ± 69.8	1025.5 ± 111.2	964.7 ± 94.5	790.2 ± 35.9	* 608.2 ± 47.2	662.4 ± 33.8	577.5 ± 21.5
5HIAA (pg/mg)		879.7 ± 27.3	893.8 ± 45.1	1259.4 ± 110.7	1045.2 ± 70.2	552.5 ± 31.8	493.4 ± 32.9	713.9 ± 66.7	618.9 ± 38.1
5HIAA/5HT		1.04 ± 0.08	0.87 ± 0.04	1.35 ± 0.17	1.17 ± 0.14	0.70 ± 0.02	* 0.82 ± 0.03	1.07 ± 0.08	1.08 ± 0.06

* denotes p < 0.05 compared with control

PPI susceptibility to the *N*-methyl-D-aspartate receptor (NMDAR) antagonist MK-801 indicates potential dysregulation of the glutamate system. To explore this possibility, we measured serum antibodies to the NMDAR subunit NR2. Intriguingly we found that infection with *T. gondii* as a juvenile lead to elevated serum antibodies to NR2 as compared with infection controls (Fig 6.3). Infection during adulthood did not elevate NR2 antibodies as compared with controls, but both groups had elevated antibodies compared to juvenile infection controls. Surprisingly the same three groups with elevated anti-NR2 antibodies had susceptibility to MK-801 (0.3 mg/kg) in PPI (Fig 6.1).

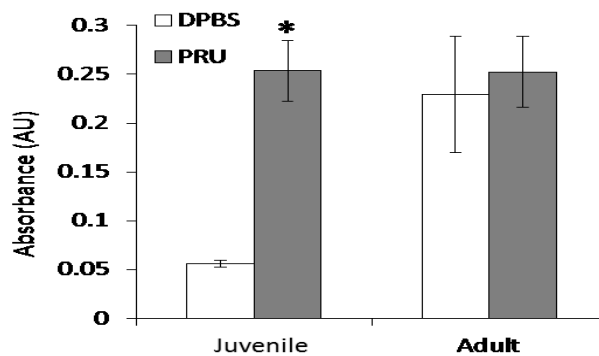


Fig 6.2 Anti-NR2 antibody in serum

Juvenile DPBS, n=10; PRU, n=18;

Adult DPBS, n=9; PRU, n=18. *

denotes $p < 0.05$

6.4 Discussion

Here we have shown for the first time that exposure to *T. gondii* during adolescence can lead to different behaviors and changes in the brain than those seen following parasite exposure during adulthood. Consistent with previous findings, we have shown that *T. gondii* infection did not alter pre-pulse inhibition (PPI) of the acoustic startle behavior in adult infected mice (134, 140). Unexpectedly, infection during pre-adolescence (juvenile) also did not alter PPI. This suggests that infection during perinatal brain development was

not sufficient to alter neuronal connections to a degree that would strongly affect rodent behavior.

Administration of psychostimulants has been shown to stimulate behavioral alterations in rodents. Here we report for the first time that administration of the psychostimulants MK-801 and amphetamine can lead to different alterations in PPI in juvenile and adult infected mice, implicating the age mice are infected on the modulation of different neurotransmitter systems.

We found that MK-801 administration led to a PPI deficit in juvenile infected mice, but PPI facilitation in adult infected mice. Indeed, PPI inhibition and facilitation are both observed in patients with schizophrenia, suggesting that rodent infection with *T. gondii* as juveniles or adults can model these two populations of patients with schizophrenia (212). Our behavioral results with MK-801 suggest juvenile and adult infection impairs the glutamate system differently. MK-801 is a specific NMDAR antagonist that can induce PPI disruption in rodents in a dopamine independent manner (213, 214). This PPI disruption is suggested to be due to the action of MK-801 on multiple brain regions and is not specific to action on the hippocampus alone (214). It is therefore plausible that differences in PPI susceptibility to MK-801 may be due to alterations in NMDAR subunit composition in multiple brain regions (e.g. decreased subunits in juvenile infected mice and increased subunits in adult infected mice).

To explore the possible contribution of NMDARs to MK-801 behavioral changes, we looked at the level of serum antibodies to the NR2 subunit of the NMDAR. Antibodies to the NMDAR have been associated with encephalitis and psychiatric disorders in patients (215). Further, the mechanistic action of MK-801 has been shown to depend on the NR2 subunit (216). Consistent with a previous report, we found *T. gondii* infection increases levels of serum antibodies to NR2 in mice (217). However, while we found elevated antibody levels as compared with controls in the juvenile infected group, antibodies were elevated in the control and infected adult groups. The anti-NR2 antibody levels in the juvenile group are consistent with the PPI deficits observed in that group. It is plausible that juvenile infection with *T. gondii* increases antibodies to NR2 that lead to a decrease in NMDARs, and consequently affect behavior.

We also found differences in PPI in juvenile and adult infected mice after amphetamine administration. While adult infected mice exhibited a deficit in PPI after amphetamine administration, there was no impairment in juvenile infected mice. Amphetamine has been shown to induce PPI deficits in different mouse strains by stimulating the release of pre-synaptic dopamine (218). Our behavior results therefore suggest differences in dopamine content due to the age of infection.

Indeed, we found a number of changes in monoamines due to *T. gondii* infection in the striatum and hippocampus of juvenile and adult-infected mice. Most striking is that juvenile infected mice show a decrease in striatal and hippocampal dopamine, while adult infected mice show a decrease in only striatal dopamine. This difference may partly

explain the behavioral change seen in adult infected mice after amphetamine administration. However, a decrease in dopamine due to *T. gondii* infection is contrary to previous findings (150, 151, 219). Differences in the mouse and parasite strain used in the studies, as well as the age at which mice were infected and how long after infection samples were collected may play a large role in discrepancies of neurotransmitter levels.

Taken together, our study shows that juvenile and adult infection with *T. gondii* can lead to different brain and behavioral changes in mice. These changes may be indicative of different mechanisms by which *T. gondii* affects neurodevelopment, and subsequently behavior. While *T. gondii* infection appears to have an overall consequence on the glutamate and dopaminergic system, our data suggests that juvenile infection may have a greater impact on dysregulation of the glutamate system, while adult infection may have a greater impact on dysregulation of the dopamine system. Future work should focus on finding the molecular targets that are modulated by infection at these different time points.

CHAPTER 7:

One Minute Ultraviolet Exposure Inhibits *Toxoplasma gondii* Tachyzoite Replication and Cyst Conversion without Diminishing Host Immune Response

Abstract

We developed a protocol to inactivate *Toxoplasma gondii* (*T. gondii*) tachyzoites employing 1 minute of ultraviolet (UV) exposure. We show that this treatment completely inhibited parasite replication and cyst formation *in vitro* and *in vivo* but retained their ability to evoke a robust IgG response in mice. We propose that our protocol can be used to study the contribution of the immune response to rodent behavioral alterations following *T. gondii* infection.

7.1 Introduction

Toxoplasma gondii (*T. gondii*) infection is known to affect rodent behavior (128, 134, 138, 169). While some behaviors, such as aversion to cat odor, are changed by all 3 *T. gondii* clonal subtypes (22), other behavioral alterations, such as novelty-induced activity, learning and memory, are to some extent dependent upon the parasite strain (134, 220). It is unclear whether brain and behavioral alterations are due to a direct consequence of *T. gondii* cysts residing in the brain, or an indirect effect of the immune response generated following *T. gondii* infection. To discriminate the contributions of direct and indirect effects of *T. gondii* infection, experimental tools are needed to generate an immune response to *T. gondii* proteins in the absence of active infection.

Tools to induce a *T. gondii* specific immune response without administration of a fully functioning live parasite already exist (221). For example, Toxovax®, a vaccine used to protect sheep, is made from a live attenuated Type I strain (222). However, since each clonal lineage of *T. gondii* up-regulates cytokines in a strain-specific manner (10), Toxovax® may not be suitable to study behavioral alterations specific to other *T. gondii* strains. Other vaccines made from single or combined purified *T. gondii* proteins (e.g. SAG1, SAG1 + GRA4) are able to stimulate an anti-*T. gondii* adaptive immune response (221), but the host immune response to single and/or denatured proteins may be different from the one induced by structurally intact tachyzoites. Consequently, we sought to develop a method to prevent *T. gondii* tachyzoite replication in the host without significantly altering the host's immune response to the parasite.

Ultraviolet (UV) irradiation has been shown to inactivate *T. gondii* tachyzoites. Current published protocols are based on a rather prolonged exposure to UV irradiation (i.e. up to 60 minutes) (223, 224), without clear demonstration that the inactivated tachyzoites are able to stimulate the host immune system in the absence of parasite replication or cyst production in rodent brains (223-227). Here we describe a new rapid and effective method for UV inactivation of *T. gondii* tachyzoites in just 1 minute. An advantage of our method is the use of standard equipment (Stratalinker®), thereby increasing the potential for reproducibility of the protocol. We demonstrate that 1 minute of UV inactivation is sufficient to completely inhibit *in vitro* parasite replication and cyst formation in the mouse brain, while still leading to a robust immune response in mice.

7.2 Materials and Methods

Serology and *in vitro* parasite detection by real-time PCR and cyst staining were performed using coded samples with the observer being unaware of their origin.

7.2.1 Parasite purification

Prugniaud (PRU) tachyzoites (\leq passage 3 *in vitro*) were cultured and purified as previously described (134).

7.2.2 UV-Inactivation of Parasites

Purified parasites (30 μ L) resuspended in Dulbecco's Phosphate Buffered Saline (DPBS) (1x) were added to an ultraviolet (UV)-transparent cuvette (Sarstedt AG & Co) and sealed with Parafilm®. The cuvette was placed horizontally in a UV Stratlinker

(STRATAGENE, UV Stratalinker 2400) and exposed to UV light for 1, 5, or 15 minutes at a power of 3,689.04 $\mu\text{J}/\text{cm}^2/\text{sec}$ for a total energy exposure of 221342.4, 1106712, or 3320136 $\mu\text{J}/\text{cm}^2$, respectively. This was accomplished by setting the timer on the Stratalinker® for 60 minutes, which keeps the emitted power constant, and stopping the countdown after 1, 5, or 15 minutes. Control 0 minute UV exposed parasites were added to a cuvette but not placed in the Stratalinker®. A schematic of the apparatus has been provided (Figure 7.1).

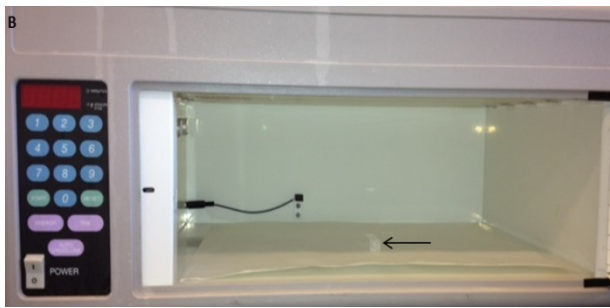
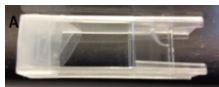


Fig 7.1 Inactivation Apparatus

A) parafilm covered cuvette containing 30 μL purified parasite.

B) Placement of cuvette (arrow) in stratalinker.

7.2.3 Trypan blue Exclusion

Live and UV-inactivated parasites were checked for damage to the cell membrane via the trypan blue dye test. Trypan blue solution (0.4% in PBS, Cellgro) was mixed with purified tachyzoites (1:1) and immediately visualized under brightfield using a Nikon Eclipse E400 microscope. Images were taken with the program EOS Utility 2.8.1.0 (Canon, EOS Rebel T1i camera).

7.2.4 Parasite Replication Assay

Purified live and UV-inactivated tachyzoites were examined for *in vitro* replication competency using a modification of an established immunofluorescence procedure (228). Tachyzoites were added to HFF cells growing in 8-chamber slides (Millipore). At 2 -9 days post-infection (37°C, 5% CO₂), cell monolayers were rinsed with DPBS, fixed, permeabilized and then immunolabeled with rabbit (Rb) anti- SAG-1 (AbD Serotec, UK) followed by Alexa Fluor 594 goat anti-Rb (red, Life Technologies). DAPI (Invitrogen) for visualizing host cell nuclei was added to the secondary antibody. Stained cells were examined by epifluorescence using a Nikon eclipse E400 microscope with the program MetaVue version 6.2r6. Prior to staining, cells were examined by phase contrast using Axiovert 100 (Carl Zeiss) and images taken with Axiovision Rel 4.8.

7.2.5 Parasite Red/Green Invasion Assay

Purified live and UV-inactivated tachyzoites were examined for *in vitro* invasion competency as previously described (228). Briefly, purified parasites were added to HFF cells growing in 8-chamber slides (Millipore). At 1 hour post-infection, cells were rinsed, fixed and then immunolabeled with Rb anti-SAG-1. Cells were then permeabilized and immunolabeled with MAb 9e11 anti-SAG1 (Argene Inc., NY, USA) followed by a mixture of Alexa Fluor 594 goat anti-Rb (red, Life Technologies) to detect attached/extracellular tachyzoites and Alexa Fluor 488 (green, Invitrogen). DAPI (Invitrogen) was added to secondary. Cells were visualized via epifluorescence as described above.

7.2.6 Animals

Male BALB/c mice, 5 weeks old (The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animal protocols were approved by the Animal Care and Use Committee of Johns Hopkins University (JHU). Mice were housed 3-5 per cage with 14.5/ 9.5 hours of light/dark cycle and free access to food and water.

7.2.7 Mouse Inoculations

Parasites were purified and UV-inactivated as described above. Alum adjuvant (Thermo Scientific) was added drop-wise to inactivated parasite (1:1) with mixing after each drop. Positive control inocula consisted of live parasites only. Negative control inocula consisted of DPBS and alum adjuvant (1:1). All solutions were placed on a rotating shaker (Barnstead Lab-Line) at medium-high speed for 40 min. Mice were injected intraperitoneally with 200 μ L of either 400 UV-inactivated tachyzoites: alum adjuvant (n=5; 2 tachy/ μ L; 1:1), 40,000 UV inactivated tachyzoites with and without alum adjuvant (n=5; 200 tachy/ μ L; 1:1); 300,000 UV-inactivated tachyzoites with and without alum adjuvant (n=5; 1500 tachy/ μ L; 1:1), 400 live tachyzoites (n=4; 2 tachy/ μ L), or mock-infected with DPBS: alum adjuvant (n=3; 1:1).

Boosters were administered 12 days post-infection. Mice first administered UV-inactivated tachyzoites with and without alum adjuvant or mock-infected with DPBS: alum adjuvant were given the same preparations as a booster. Mice first given live parasite were administered DPBS only as a booster.

7.2.8 Serology

Blood was collected via tail bleed and then centrifuged at 10,000 x g for 10 minutes on a table top centrifuge to separate cells from serum. Anti-*T. gondii* IgG was measured in sera using ELISA kits from IBL America (Minneapolis, MN, U.S.A.) modified as previously described (174). The primary antibody consisted of diluted serum (1:100) and secondary antibody was enzyme labeled anti-mouse IgG.

7.2.9 Parasite Detection in Mouse Brain

Mice were sacrificed 4 weeks after initial infection, whole brains were excised, and then washed with cold 1x PBS. Brains were homogenized by serial passage through 18, 20, and 23 gauge needles in sequential order, and then centrifuged at 3,300 RPM for 5 minutes at room temperature. The pellet was resuspended in 1 mL PBS and divided for 1) parasite cyst staining, 2) parasite DNA detection by real-time polymerase chain reaction (RT-PCR) and 3) parasite viability.

7.2.9.1 Cyst Staining

Twenty percent of brain homogenate was stained with Fluorescein labeled Dolichos Biflorus Agglutinin (DBA) (1:200; Vector Laboratories) to visualize parasite cysts. Stained brain homogenates were examined using Nikon eclipse E400.

7.2.9.2 Real-time Polymerase Chain Reaction

Thirty percent of brain homogenate was used for TaqMan real-time PCR analysis. DNA was extracted from 3 samples of brain homogenate per mouse (10% homogenate/ mouse)

using a standard kit (MOBio Tissue Kit, Carlsbad, CA, U.S.A.). Real-time PCR was performed for the unique *T. gondii* 5S rRNA gene (229) using the following primers and probe: 5'-TGC TGC GTT CTT CAT CGT-3' forward, 5'-TGC TTT CAG ATT GCT TCC TAA A-3' reverse, 56-FAM/CGC GAG CCA AGA CAT CCA TTG C/36-TAMSp. Mouse beta-actin (Mm00607939 s1, Life Technologies) was run as a housekeeping gene/loading control. Each sample was run in triplicate with 100 ng DNA. The PCRs were performed on an ABI7900HT real-time PCR system (Applied Biosystems, Foster City, CA). Real-time results were analyzed using SDS 2.4 software (version 2.4; Applied Biosystems).

7.2.9.3 Viable Parasite Recovery

Fifty percent of brain homogenate was used to purify brain cysts. To separate parasite cysts from brain cell debris, isotonic percoll was added to brain homogenate and centrifuged at 2,400 RPM for 15 minutes at room temperature. The pellet was resuspended in DPBS (1X) and centrifuged at 3,300 RPM for 10 minutes at room temperature. The remaining pellet was resuspended in HFF medium and plated in duplicate on HFF cells cultured on 8-chamber microscope slides (Millipore). After 2 weeks, cells were washed, fixed, permeablized, stained, and images taken as described under the replication assay section.

7.2.10 Statistical Analysis

Data were first tested for normality using IBM SPSS Statistics 21. If passed, one-way analysis of variance (ANOVA) with Bonferroni post-hoc was run with infection as the

independent variable. If failed, data were subjected to a Kruskal-Wallis one-way analysis of variance by ranks with infection as the independent variable. Mann-Whitney U-test was then run to compare specific infection groups at individual time points; $p < 0.05$ was considered significant.

7.3 Results

We first sought to determine whether UV exposure affects the integrity of the tachyzoite cell membrane via the trypan blue dye test. We found that, similar to live parasites, purified *T. gondii* tachyzoites exposed to UV for 1 minute were impermeable to vital dye trypan blue whereas longer exposures, i.e. 5 and 15 minutes, resulted in immediate intracellular accumulation of the dye (Fig 7.2). We then checked whether the replication of 1 minute UV-exposed tachyzoites was compromised. While live tachyzoites successfully replicated and eventually lysed host HFF cells, 1 minute UV-exposed tachyzoites were unable to replicate (Fig 7.3). As replication may have been affected by the inability to invade HFF cells, we performed an invasion assay with 1 minute UV-exposed tachyzoites. We found that 1 minute UV treatment did not affect the ability of tachyzoites to invade HFF cells (Fig 7.3, inset). Based on these results, we selected 1 minute UV-exposed tachyzoites to study *in vivo*.

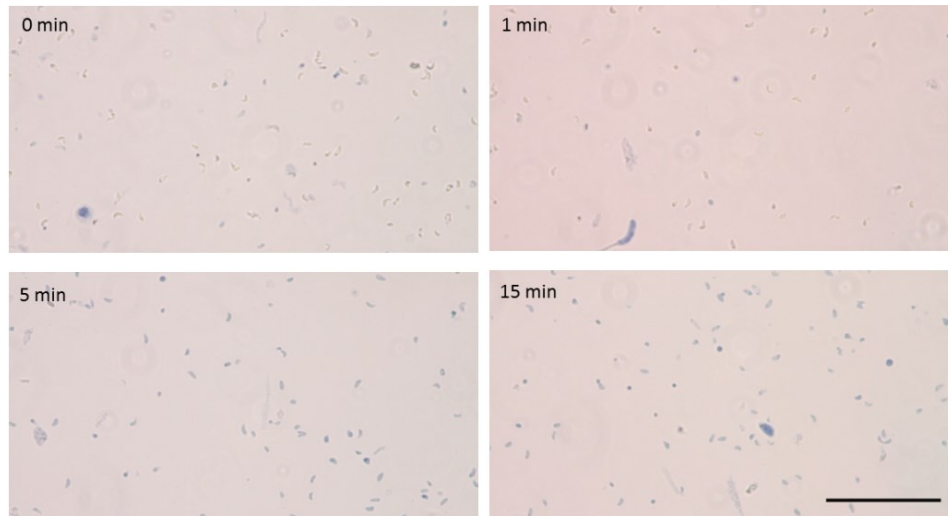
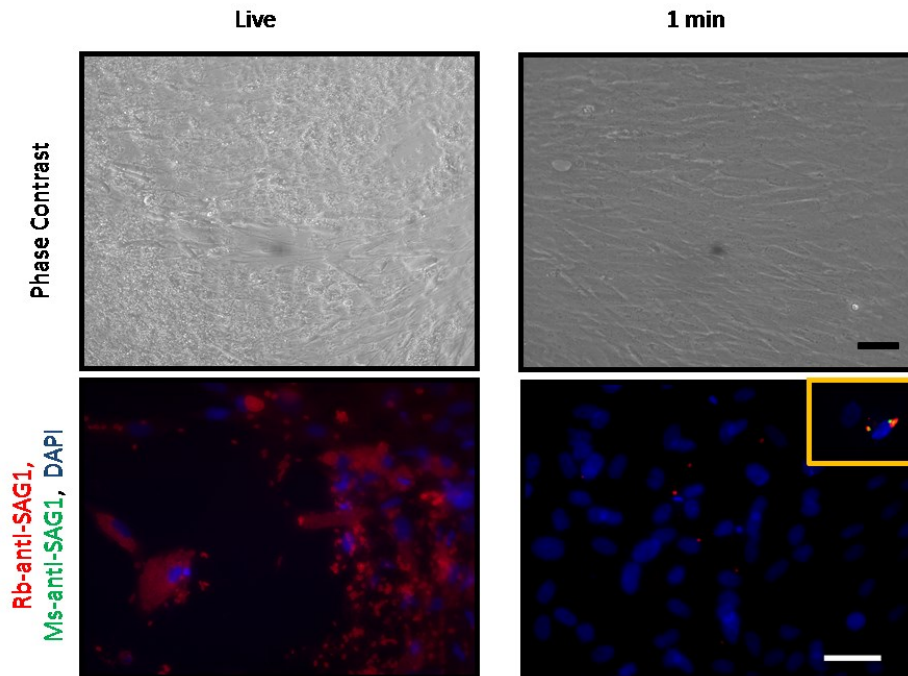


Fig 7.2 TrypanBlue Exclusion of UV inactivated tachyzoites

Purified tachyzoites exposed to UV for 0 or 1 min remain clear in the presence of TrypanBlue. Tachyzoites exposed to UV for 5 or 15 min are immediately stained blue. Scale bar represents 50 μ M.



7.3 UV exposure prevents *in vitro* tachyzoite replication

HFF cells were infected with either live tachyzoites or tachyzoites exposed to UV for 1 min and then examined microscopically after 9 days. As shown by both phase contrast and immunofluorescence, live tachyzoites replicated normally, resulting in cell lysis. In contrast, UV-exposed tachyzoites, although able to penetrate the host cells (inset), were unable to replicate, leaving only sparse single tachyzoites.

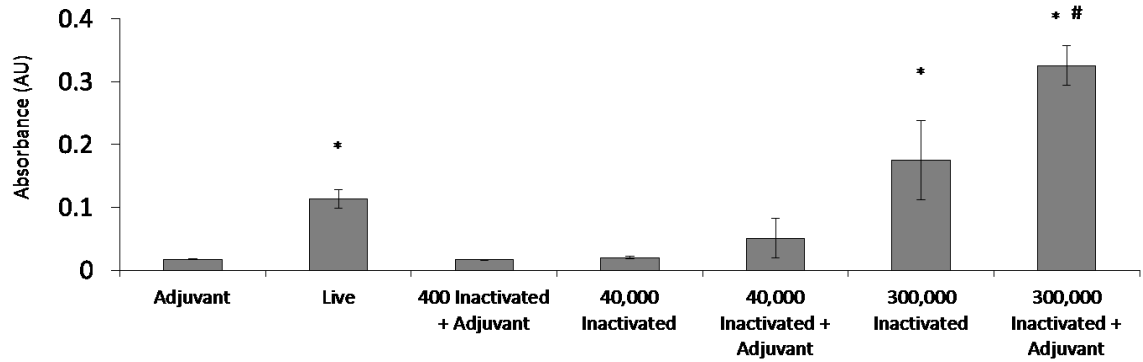
Inset: Red/ green Invasion Assay. See Methods section for details.

Red and green: Immunofluorescent staining of *T. gondii* tachyzoite surface antigen 1 (SAG1)

Blue: Nuclear staining with DAPI

Scale Bars: 50 μ M

We first optimized the administration condition and dose of inactivated tachyzoites to elicit a robust anti-*T.gondii* IgG response in mice. As expected, injection of 400 live tachyzoites produced a significant increase in anti-*T. gondii* IgG levels compared to injection with adjuvant. We then tested several doses of inactivated parasites with or without adjuvant to determine the optimal dose to elicit a robust anti-*T. gondii* IgG response. Neither 400 nor 40,000 inactivated tachyzoites with or without adjuvant significantly elevated levels of anti-*T.gondii* IgG compared to adjuvant only ($p>0.05$). However, injections of 300,000 inactivated tachyzoites with or without adjuvant led to a significant rise of anti-*T.gondii* antibodies as compared to adjuvant ($p<0.05$) (Figure 7.4). The greatest anti-*T.gondii* IgG response was produced by administration of 300,000 inactivated parasites with adjuvant compared to that following injection of 400 live tachyzoites or 300,000 inactivated parasites without adjuvant (all $p<0.05$). We therefore selected the dose of 300,000 inactivated tachyzoites with adjuvant to be given to mice (inactivated group) to compare with mice given live parasite (live group) or adjuvant only (adjuvant group).



7.4 Anti-Toxoplasma IgG Levels at 20 days post infection (dpi)

Live and 300,000 inactivated tachyzoites with and without adjuvant significantly elevated anti-Toxoplasma IgG levels in mice 20 dpi. There was a significant group effect [H=23.715, 6 d.f., $p = 0.0001$]. Post-hoc comparisons showed that the antibody levels were significantly higher in live, 300,000 inactivated, 300,000 inactivated with adjuvant groups vs. adjuvant control and 300,000 inactivated with adjuvant vs. live. * denotes $p < 0.05$ relative to adjuvant control. # denotes $p < 0.05$ relative to live parasites. Error bars denote SEM. Adjuvant, $n=3$; live, $n=4$; 400 inactivated + adjuvant, $n=5$; 40,000 inactivated, $n=5$; 40,000 inactivated + adjuvant, $n=5$; 300,000 inactivated, $n=5$; 300,000 inactivated + adjuvant, $n=5$.

In order to better characterize the response to different treatment conditions, we looked at the kinetics of the anti-Toxoplasma IgG levels and mouse body weight as a clinical symptom of sickness. We found that at 20 dpi anti-Toxoplasma IgG levels were significantly higher in the inactivated group compared to the live or adjuvant group ($p < 0.05$). However, this difference between the inactivated and live group was resolved

by 30 dpi ($p>0.05$) (Fig 7.5). Despite the presence of relatively high anti-Toxoplasma IgG levels in mice of the inactivated group, these animals did not significantly lose weight as measured at 0, 10, 20 or 30 dpi (all $p>0.05$). In contrast, mice of the live group had significantly lower body weights compared to mice of the adjuvant or inactivated group at 10, 20, and 30 dpi ($p<0.05$) (Fig 7.6).

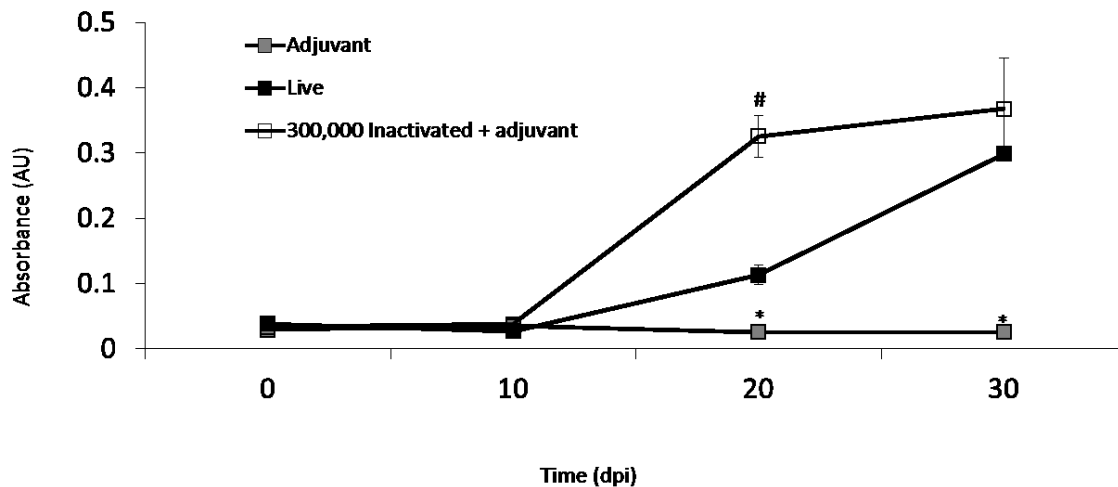


Fig 7.5 Kinetics of Anti-Toxoplasma IgG Levels

Live and inactivated tachyzoites elicit increased IgG levels in mice 20 and 30 dpi. There was a significant group effect at 20 and 30 dpi [$H=9.726$ and 7.477 , 2 d.f., $p=0.008$ and 0.024 , respectively]. Post-hoc comparisons showed antibody levels were significantly lower in the adjuvant group vs. live and inactivated groups at 20 and 30 dpi; inactivated IgG levels were higher at 20 dpi vs. live. * denotes $p<0.05$ relative to live and inactivated groups; # denotes $p<0.05$ relative to live. Error bars denote SEM. Adjuvant, $n=3$; Live, $n=4$; 300,000 inactivated + adjuvant, $n=5$.

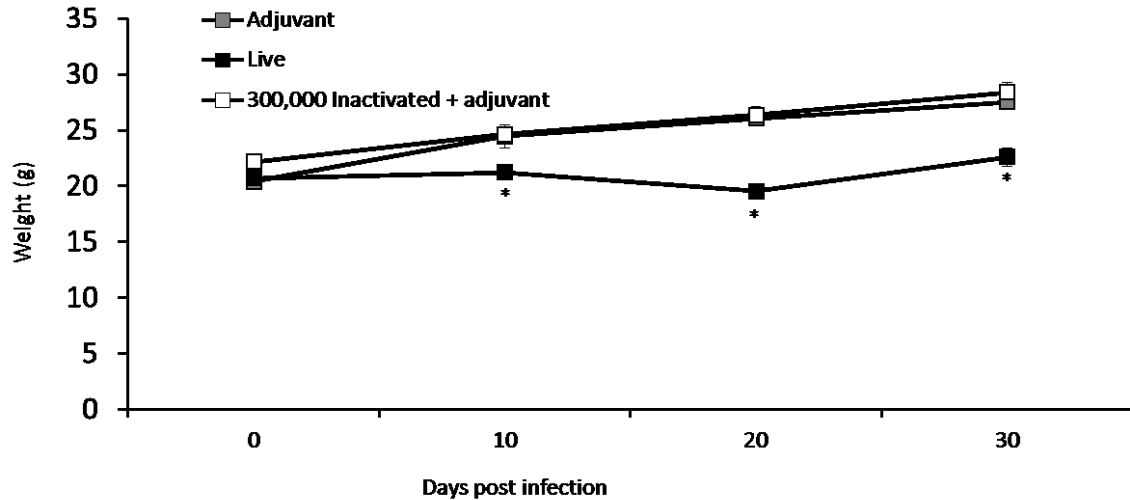


Fig 7.6 Kinetics of mouse weight

Live-infected mice weighed significantly less than adjuvant controls and 300,000 inactivated parasite with adjuvant group at 10, 20, and 30 dpi. Adjuvant, n=3; live, n=4; inactivated, n=5, * denotes $p \leq 0.05$ as compared to adjuvant and inactivated administered mice. Error bars denote SEM.

We then determined whether the IgG response in mice of the inactivated group was associated with viable *T. gondii* presence in the brain 30 dpi. Viable parasites were found in the brain homogenates from mice of the live group but were absent in the homogenates from mice of the inactivated group (Fig 7.7). We also looked for the existence of *T. gondii* cysts. Parasite cysts were found in the brains of mice of the live group (n=3) but not in the brains of mice of the inactivated group (n=5) (Fig 7.7A). To confirm the absence of viable parasites in mice of the inactivated group, their brain homogenates were added to HFF cultures. No *T. gondii* tachyzoite growth was observed from the brains of mice given inactivated parasites. In contrast, there was a robust growth of the

parasite from the brains of mice given live tachyzoites (Fig 7.7B). To further verify that inactivated tachyzoites were not present in the mouse brains, we checked for *T. gondii* DNA via real-time PCR. *T. gondii* specific 5S rRNA genomic DNA was detected in brain homogenates of mice given live parasites, but not in animals given inactivated parasites or adjuvant only. Regardless of treatment conditions, the host specific housekeeping gene/ loading control Beta-actin was observed in all samples and the levels did not differ between groups ($p > 0.05$) (Fig 7.8).

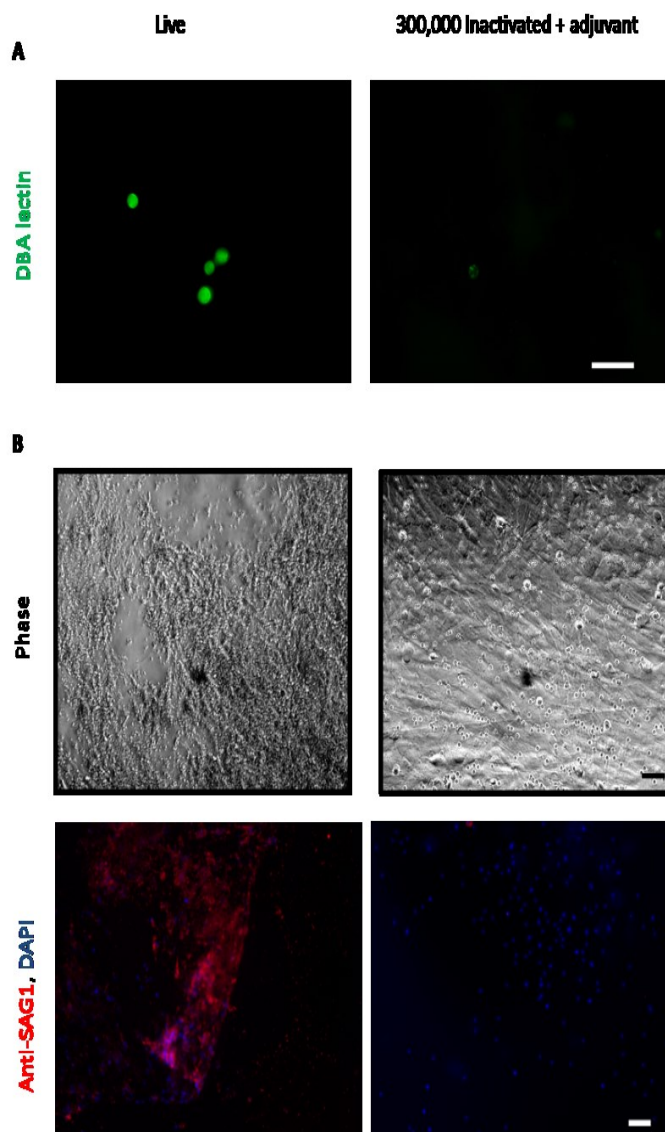


Fig 7.7 Parasite cyst presence and parasite viability from mouse brain

A) Parasite cyst DBA lectin (green) seen in brain homogenates of live parasite administered mice only.

B) Parasite tachyzoites grew on HFF cells from brain homogenate of mice administered live parasite but not inactivated parasite.

Red is immunofluorescent staining of tachyzoite SAG1; Blue is DAPI staining of nuclei; Scale bars denote 50 μ M.

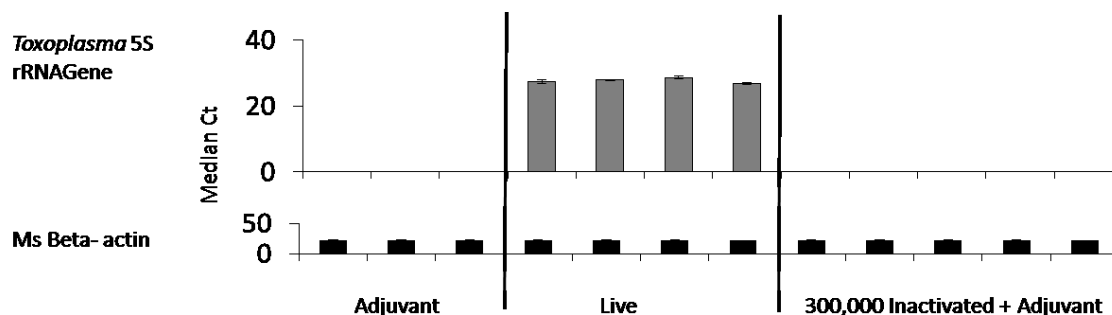


Fig 7.8 Real-time PCR of Toxoplasma 5S rRNA gene

Brain homogenates only from mice given live *T. gondii* had detectable levels of parasite DNA 30 dpi. No parasite DNA was detected in the brains of adjuvant controls or inactivated parasite administered mice. Beta-actin loading control was detected in all samples. There was no significant group affect in Beta-actin levels [$F(2,9)=0.206$, $p=0.817$]. Adjuvant, $n=3$; live, $n=4$; inactivated, $n=5$.

7.4 Discussion

The present study indicates that our method of inactivating tachyzoites with 1 minute UV-exposure results in a complete inhibition of tachyzoite replication *in vitro* and absence of viable parasites *in vivo*. Nonetheless, intraperitoneal injection of UV-inactivated parasites triggered a robust IgG immune response in mice. These results suggest that our method of inactivating *T. gondii* tachyzoites with UV could aid in investigating the role of *T. gondii*-specific immune activation on rodent behavior by effectively dissociating direct and indirect effects of the infection.

Our data are largely consistent with prior studies that utilized UV inactivation of *T. gondii* tachyzoites (223-227). For example, we also showed that tachyzoites exposed to UV were able to exclude Trypan blue, suggesting irradiation did not damage the tachyzoite membrane (225). We found that 1 minute UV-exposed tachyzoites did not replicate *in vitro*, as has been seen by others using UV inactivation of tachyzoites (225, 226). In addition, we report that mice administered inactivated parasites had a robust serological IgG response without a decrease in body weight, as has been previously published (223, 227).

In contrast to Grimwood and associates who reported that irradiated tachyzoites can infect BHK cells at a rate similar to live tachyzoites (225), we found only a few UV inactivated tachyzoites capable of binding and invading HFF cells. It is possible that similar rates of infection between live and UV inactivated parasites were not observed in our studies due to differences in the cell type used. It is also possible that we did not see many inactivated parasites at 9 dpi due to parasite degradation in culture. Indeed, it has been shown that inactivated parasites are degraded in macrophage cultures within 48 hrs of infection (226).

In contrast to prior studies used UV inactivation, we performed a more thorough validation of our method. We confirmed that inactivated parasites were not present in the mouse brain 30 dpi as tested with parasite cyst staining, culturing, and real-time PCR. However, even if we did not detect any live parasites in the brains of mice from the inactivated group at 30 dpi, one cannot completely rule out that a few parasites might

have entered the brain but were subsequently cleared. Notably, a transient presence of *T. gondii* in the brain has been suggested to contribute to behavioral abnormalities (22).

There are several caveats to our study. While the anti-Toxoplasma IgG response to the inactivated parasites lasted up to 1 month post infection in vivo, the complete time course of this response has not been evaluated. Also, whether the peripheral IgG response is associated with or followed by immune activation in the brain has not been assessed. Importantly, such a phenomenon has been seen in other models. For example, peripheral administration of BCG vaccine to rodents leads to immune activation (e.g. up-regulation of cytokines) in the brain (230).

There are a number of potential benefits to using the UV inactivation protocol described here to study the role of the host immune response to *T. gondii* infection in behavioral abnormalities. First, in contrast to synthetic immunostimulators such as poly I:C and LPS (TLR3 and 4 ligands, respectively), our protocol permits determining the immune response specific to *T. gondii* or even a particular *T. gondii* strain as the protocol employs structurally intact parasites. In this way, there would be greater construct validity to animal models of behavioral alterations following *T. gondii* infection (220, 231). Second, our method is faster than previously reported ones that take up to 60 minutes of UV exposure (223-227). Third, our protocol uses a standard piece of laboratory equipment, which has the potential to increase reproducibility of the data between research groups.

7.5 Conclusion

Here we describe a rapid and straightforward method to reliably inactivate *T. gondii* tachyzoites and stimulate an immune response *in vivo*. The inactivated tachyzoites are incapable of replicating *in vitro* and *in vivo*, or forming cysts *in vivo*, but elicit a strong serological anti-*Toxoplasma* IgG response in mice. This methodology can be implemented to study the role of immune activation following *T. gondii* infection in rodent behavioral alterations. In particular, since this method is applicable to the inactivation of any *T. gondii* strain, differences in parasite-strain immune activation can be studied.

CHAPTER 8:

Immune Response to *Toxoplasma gondii* Infection Affects Mouse Behavior

Abstract

The protozoan parasite *Toxoplasma gondii* (*T. gondii*) has been shown to modulate behavior. It has long been thought the parasite does this during chronic infection as parasite cysts persist in the brain. Here we show that behavioral modifications of *T. gondii* infected mice may actually be due to the host immune response to infection, rather than the parasite cyst. We show that mice administered live *T. gondii* (able to produce cysts) and inactivated *T. gondii* (unable to produce cysts) behave similarly in their response to the NMDAR antagonist MK-801 in pre-pulse inhibition of the acoustic startle and cued memory in fear conditioning. In contrast, live and inactivated *T. gondii*-infected mice show opposite behaviors of anxiety in the dark-light box and possible contextual memory deficits in fear conditioning, as well as associative memory in passive avoidance. These findings provide evidence that the immune response to *T. gondii* modulates rodent behavior.

8.1 Introduction

Toxoplasma gondii (*T. gondii*) is a protozoan parasite found world-wide that can infect any warm-blooded mammal, including humans. Epidemiological studies implicate infection with this parasite in schizophrenia and bipolar disorder (6, 129), as well as other changes in behavior (5, 15, 17). Rodent behaviors have also been shown to be altered by *T. gondii* infection (128, 134, 138, 141, 169, 220). Yet how infection with *T. gondii* alters behavior is not understood.

One premise is that the continued presence of *T. gondii* cysts in specific brain regions can affect behavior. Indeed, *T. gondii* cysts have been shown to contain tyrosine hydroxylase homologs and produce dopamine, a neurotransmitter associated with many behaviors (20, 149). Parasite cysts localized in two brain regions (amygdala and forebrain) have been associated with predator odor avoidance and anxiety in rodents (138, 232). However, other rodent studies were unable to find any regional preference of parasite cysts in the brain (233), and there are currently no reports on detection of *T. gondii* cysts in the brain of patients with psychiatric disorders. Thus, it is possible that *T. gondii* cyst regional localization may not be the sole factor contributing to behavioral alterations.

The host immune response to an infection may also contribute to behavioral changes. Studies have revealed that peri- and post-natal infection with bacterial and viral mimics (LPS and PolyIC, respectively) is sufficient for altering rodent behavior (94, 234, 235). Similarly, changes in rodent behaviors have been seen upon administration of bacillus Calmette-Guerin (BCG), an attenuated *Mycobacterium bovis* (230). Furthermore, one

study thus far has shown that decreased aversion to cat odor in *T. gondii* infected rodents does not require parasite persistence (22). It is therefore plausible that the host immune response to *T. gondii* infection, rather than the persistence of parasite cysts, plays a large role in the development of behavioral modifications.

In this study, we explore the contribution of the host immune response to *T. gondii* infection in altering rodent behavior, with an emphasis on behaviors related to human psychiatric disease. Due to parasite strain-specific behavioral changes (134) and cytokine responses (236), we utilize a parasite inactivation method previously developed (Chapter 7), enabling the use of the same *T. gondii* strain for the live and inactivated parasite infections. Here we report new evidence that the immune response to *T. gondii* infection may be sufficient to modify behavior.

8.2 Materials and Methods

8.2.1 *T. gondii* tachyzoite purification and UV-inactivation

Prugniaud (PRU) tachyzoites (< passage 4 *in vitro*) were cultured and purified as previously described (134). Purified tachyzoites were inactivated for 1 minute using a UV Stratalinker (STRATAGENE, UV Stratalinker 2400) under conditions explained elsewhere (Chapter 7).

8.2.2 Mouse Inoculations

Male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were housed 5 per cage with 14.5/ 9.5 hours of light/dark cycle and free access to food and water in the Johns Hopkins University (JHU) animal facility. Mice were injected at 5 weeks of age intraperitoneally with either 200 μ L Dulbecco's Phosphate Saline (DPBS), live PRU tachyzoites (400 tachyzoites/ 200 μ L), alum adjuvant diluted in DPBS (1:1) (Invitrogen), or inactivated PRU tachyzoites mixed with alum adjuvant (1:1) (Invitrogen; 300,000 tachyzoites/ 200 μ L). Prior to injections, inocula were placed on a shaker for 45 minutes as previously described (Chapter 7). Booster injections were administered 12 days post infection (dpi), with DPBS and live PRU- infected mice now administered DPBS, adjuvant mice now administered adjuvant, and inactivated PRU tachyzoites (300,000 tachyzoites/ 200 μ L) with alum adjuvant given to mice first given inactivated parasites. The Animal Care and Use Committee of JHU approved all protocols.

8.2.3 Behavior Analysis

Mouse behavior was tested between 2 and 4.5 months post infection (mpi) in the following order: open field, pre-pulse inhibition of the acoustic startle (PPI) with saline administration, PPI with 0.1 mg/kg and 0.3 mg/kg MK-801 administration, dark-light box, passive avoidance, and fear conditioning. All testing was conducted between the hours of 3 and 8:30 pm. Data was normalized to infection controls for tests that showed differences between adjuvant and DPBS controls.

8.2.3.1 Novelty-induced activity of the open field

Novelty induced activity was measured in the open field (San Diego Instruments) in 5 min bins, over a 30 min trial. Central, peripheral, and rearing beam breaks were automatically recorded. The periphery was designated as 2.5 cm on all sides of the chamber. Central and peripheral beam breaks were combined to give a measure of total activity. Beam break activity in the periphery and center were translated into the time spent in each location using the PAS Software (San Diego Instruments).

8.2.3.2 Sensorimotor gating of pre-pulse inhibition of the acoustic startle

Sensorimotor gating was tested using pre-pulse inhibition of the acoustic startle (PPI). Briefly, 5 minutes prior to testing, mice were injected intraperitoneally with 200 μ L bacteriostatic 0.9% sodium chloride (Saline) (Hospira, INC; Lake Forest, IL) or 0.1 mg/kg or 0.3 mg/kg (+)-MK801 hydrogen maleate (Sigma-Aldrich). In the testing apparatus (San Diego Instruments), mice were acclimatized to a 76dB background noise for 5 min. They were then given 10 presentations of a 120dB pulse and 10 presentations of no pulse. This was followed by 5 presentations in randomized order of a 120 dB pulse, no pulse, or pre-pulses with a pulse (77, 78, 80, 84, 88 dB- 120 dB). The intervals between each presentation varied from 10-19 seconds. Mean PPI % was calculated by averaging all PPI% values for presentations of all pre-pulses for each experimental group.

8.2.3.3 Dark-light Box

Anxiety was evaluated using the dark-light box (Colbourn). Mice were placed in the transparent side and allowed to freely move for 5 min between the dark and light

chambers. The latency for i) each crossing, ii) total time spent on each side, and iii) the number of crosses were automatically recorded using Graphic State v 3.03.

8.2.3.4 Fear Conditioning

Context and cued (tone)-dependent learning and memory were evaluated with fear conditioning (Colbourn) over a 3-day trial. All chambers were in custom made soundproof enclosures. On day 1, mice were placed in chambers for a total of 5 min. At 2 min, an 85 dB, 2000 Hz tone was played for 30 seconds. During the last 2 seconds of the tone presentation, a 1.0 mA shock (Med Associates, Inc.) was delivered. The following day, day 2, contextual freezing was tested. Mice were placed in the same chamber in which they were previously shocked. On day 3, cued (tone) dependent freezing was tested. Mice were placed in an opaque box that was then placed in the fear conditioning chamber for 5 min. At 2 min, mice were presented with an 84 dB-tone, 2000 Hz that lasted 3 min. On each day, freezing (%) was automatically recorded in 1 min bins (Freezescan, CleverSys Inc).

8.2.3.5 Passive Avoidance

Associative learning and memory was evaluated using a 2-day passive avoidance test (San Diego Instruments). On the first day, training phase, mice were placed in a lit compartment with the gate to the dark compartment shut. After 30 seconds, the gate opened allowing the mouse to cross to the dark compartment. Once the mouse crossed over, the gate automatically closed and after a 3 second delay a 0.3 mA shock was administered for 3 seconds. Twenty-four hours later, during the testing phase, the mouse

was placed once again in the lit compartment with the gate to the dark compartment shut. After 5 seconds, the gate opened allowing the mouse to freely move to the dark compartment. The trial ended either when the mouse crossed to the dark compartment, or once 10 min elapsed. For each phase, the latency in seconds for the mouse to cross from the light to dark compartment was automatically recorded.

8.2.4 Statistical Analysis

Data was first checked for normality and equal variance using SPSS (v.21). If failed, either non-parametric Kruskal-Wallis with multiple comparisons or Mann-Whitney U test was performed to compare DPBS and live, adjuvant and inactivated, and live and inactivated infection groups. A $p \leq 0.05$ was considered significant.

8.2.5 Infectivity Confirmation

Blood was taken at the culmination of behavioral testing and centrifuged at 10,000Xg to collect serum. Total anti-Toxoplasma IgG was measured via enzyme-linked immunosorbent assay (ELISA) as described previously (174).

8.3 Results

We evaluated the role of live *T. gondii* infection and the immune response to *T. gondii* infection on the manipulation of mouse behavior. To do so, we infected mice with either live *T. gondii* tachyzoites (“live mice”), which can replicate and produce parasite cysts in the brain, or inactivated *T. gondii* tachyzoites (“inactivated mice”), which are unable to replicate or produce parasite cysts. We first looked at novelty-induced activity in the open

field as an indicator of general activity level and possible anxiety. Infection with either live or inactivated *T. gondii* did not change total, central, peripheral, or rearing activity as indicated by no significant difference in the number of beam breaks or total time spent in each zone as compared with controls (data not shown).

While anxiety and overall activity were unchanged in the open field, mice administered live and inactivated *T. gondii* exhibited altered anxiety levels in the dark-light box. Live *T. gondii* infected mice were more anxious than infection controls and inactivated mice, while live mice exhibited decreased anxiety (Fig 8.1). Live parasite-infected mice spent greater amount of time in the dark chamber during the first crossover as compared with controls and inactivated mice. However, over the entire 5 min trial, inactivated mice spent less time in the dark as compared with live mice, and both parasite administered mice spent more time in the dark compared with controls (Fig 8.1A, B). Also, live mice had transitioned a fewer number of times between the dark and light chambers while inactivated mice had greater number of transitions (Fig 8.1C). Taken together, the data reveals that live *T. gondii* infection increases anxiety in mice, while the immune response alone (inactivated parasite) decreases anxiety.

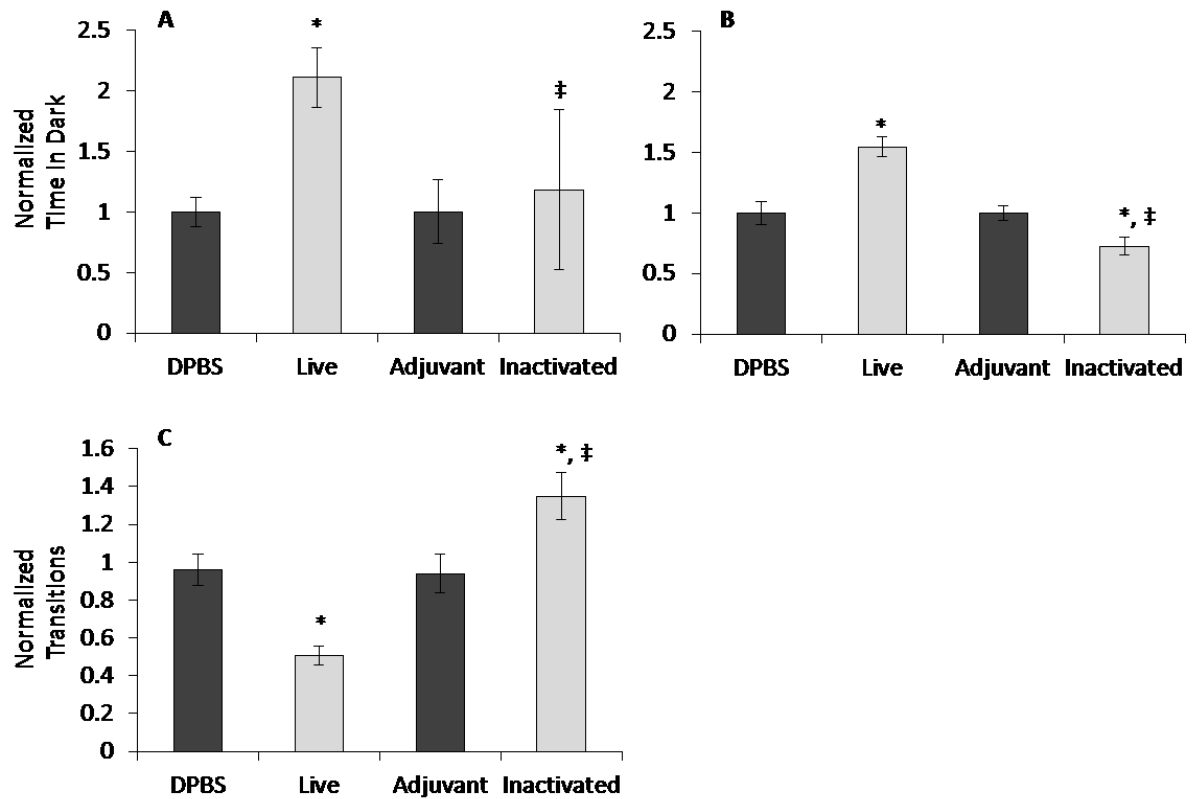


Fig 8.1 *T. gondii* infection increases mouse anxiety in the dark-light box

A) Normalized time it first took mouse to transition from dark to light chamber

B) Normalized time mouse spent in dark over entire trial

C) Normalized total number of transitions

DPBS, n=9; Live, n=9; Adjuvant, n=7; Inactivated, n=16.

* denotes $p < 0.05$ compared to control, ‡ denotes $p < 0.05$ compared to live

We also checked the effect of *T. gondii* infection and the immune response to *T. gondii* infection on two learning and memory tasks: passive avoidance and fear conditioning. We found no associative learning and memory impairment in the passive avoidance test (Fig 8.2) or fear conditioning (Fig 8.3). In the passive avoidance task, there was no difference in the amount of time it took for live and inactivated mice to cross to the dark chamber on training day as compared with controls (Fig 8.2A). There was also no significant difference between groups in the ratio of testing to training latency (Fig 8.2B). However, inactivated mice trended towards a greater testing to training ratio as compared with live mice ($p < 0.1$), suggesting with a greater number of mice, we may be able to see an associative learning and memory deficit in the passive avoidance task due to live *T. gondii* infection.

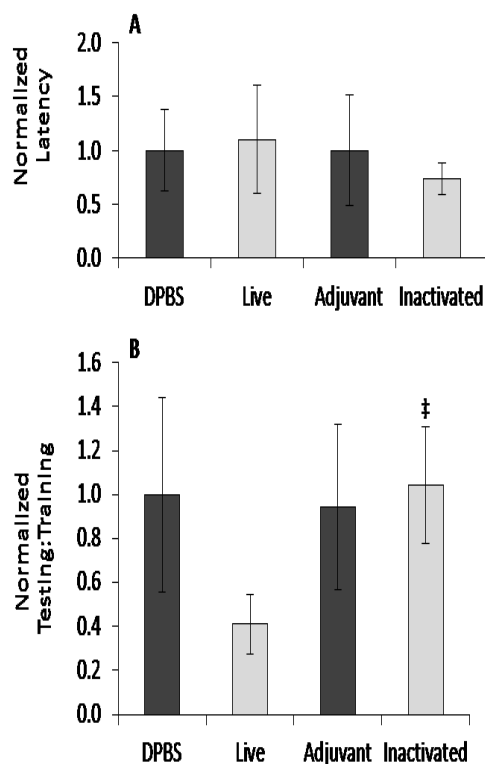


Fig 8.2 Associative Learning and Memory Impairment in *T. gondii* Infected Mice

A) Normalized latency to cross over to dark side during training

B) Ratio of testing latency: training latency

DPBS, n=10; Live, n=9; Adjuvant, n=7;

Inactivated, n=15. ‡ denotes $p < 0.1$ compared with live.

In the fear conditioning task, there was no difference between all groups on training day in the percent of time the mice froze (Fig 8.3A). There was also no difference in contextual memory, as the percent of time the mice froze was comparable between all groups. While there appears to be delineation between mice given adjuvant or DPBS as the vehicle, this was not statistically significant (Fig 8.3B). Similarly, there was no significant difference between the infected groups and controls in cued memory, but now the live and infected mice are more closely grouped together in less percent freezing, while the controls trended to freezing more (Fig 8.3C). Taken together, this data suggests there may be a deficit in cued memory in mice administered live or inactivated *T. gondii*.

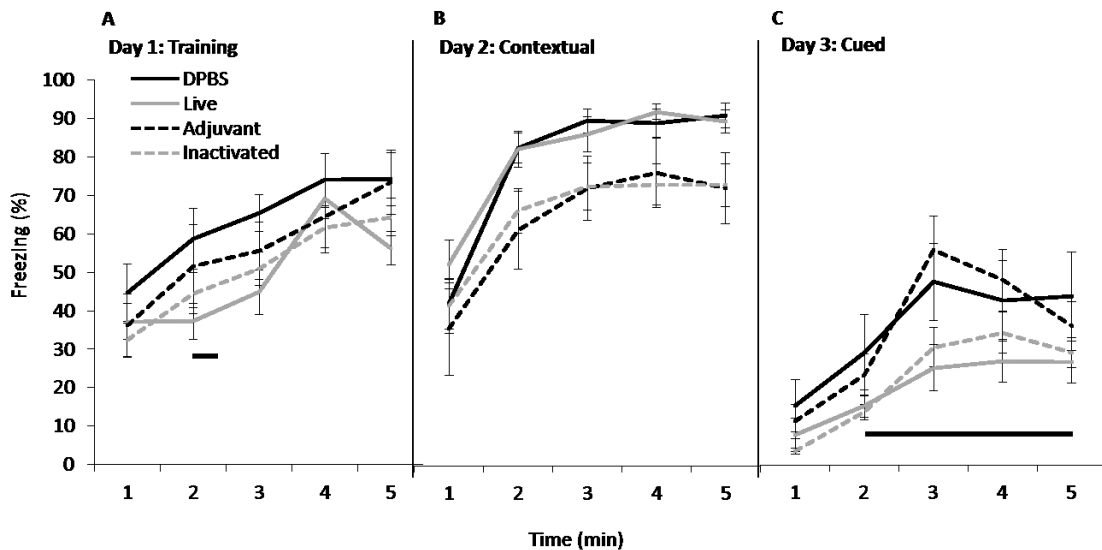


Fig 8.3 Contextual and Cued Fear Conditioning is not affected by *T. gondii* Infection

Solid horizontal line denotes time and duration the tone (84 dB, 2000 Hz) was delivered.

DPBS, n=9; Live, n=9, Adjuvant, n=7; Inactivated, n=16

As cognitive impairments may be due to problems with information processing, we looked at the effect of live and inactivated *T. gondii* administration on pre-pulse inhibition (PPI) of the acoustic startle (Fig 8.4 and 8.5). As infection with either live or inactivated *T. gondii* did not elicit deficits in PPI as compared with infection controls (Fig 8.4), we wished to determine whether infection would lead to differences in susceptibility to the *N*-methyl-*D*-aspartate receptor (NMDAR) antagonist, MK-801. Increasing doses of MK-801 lead to increased startle in DPBS, live, and inactivated mice ($p < 0.01$), but not adjuvant mice, as compared with the drug controls (saline) in each group (Fig 8.4A). Surprisingly, increasing doses of MK-801 lead to deficits in PPI for all infection groups: DPBS, live, adjuvant, and inactivated, compared to saline drug controls for each infection group (Fig 8.4B). In addition, administration of 0.3 mg/kg MK-801 lead to PPI deficits for all infection groups as compared with saline and 0.1 mg/kg MK-801 administration (Fig 8.4B). Surprisingly, when all infection groups with 0.3 mg/kg MK-801 were compared, we found that live and inactivated mice showed facilitated PP as compared with either infection controls, but did not differ from one another (Fig 8.4B). Taken together, this data suggests that the immune response to *T. gondii* infection will lead to a similar PPI response to MK-801 as infection with live *T. gondii*.

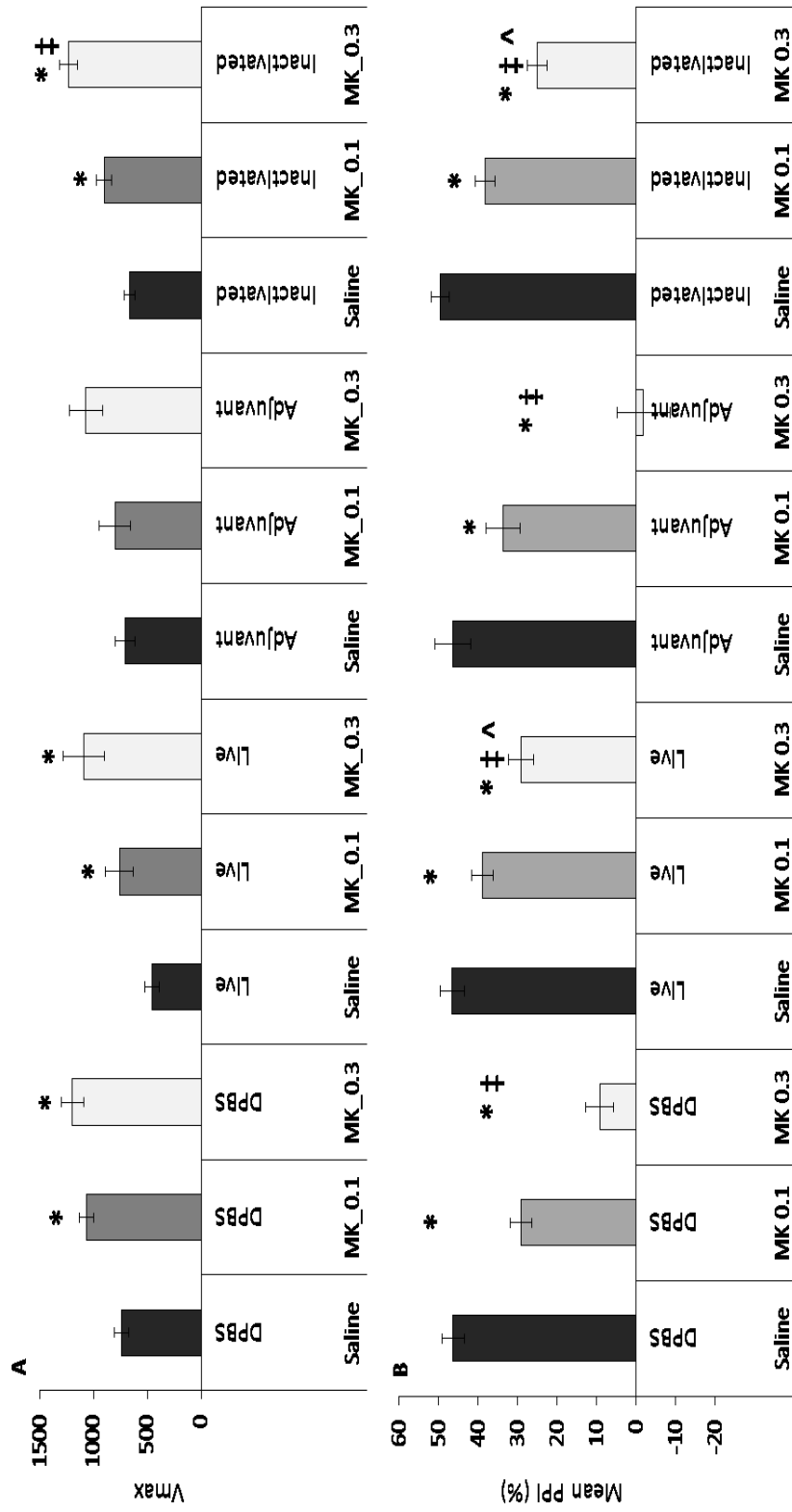


Fig 8.4 MK-801 Disruption in pre-pulse inhibition of the Acoustic Startle. A) Startle response with 0.1 and

0.3 mg/kg MK-801 administration; B) Mean PPI% with 0.1 and 0.3 mg/kg MK-801 administration. DPBS, n=10;

Live, n=9; Adjuvant, n=7; Inactivated, n=16. * denotes $p < 0.01$ compared to saline; † denotes $p < 0.01$ compared to MK 0.1 mg/kg; ^ denotes $p < 0.05$ compared to infection control (DPBS or adjuvant) with MK 0.3 mg/kg.

Because body weight differences may influence behavior, we monitored mouse weights over the course of the experiment. There was no significant weight difference between all groups during initial infection (0 days post-infection; dpi), at the beginning of behavior testing (60 dpi), or at the culmination of behavior testing (128 dpi) (Table 8.1). We also evaluated serum anti-Toxoplasma IgG at the end of behavior testing to determine whether differences in antibody levels, which suggest infectivity, may explain behavioral differences. We found there was a significant elevation of serum anti-Toxoplasma IgG due to infection with live and inactivated parasites as compared with controls ($p < 0.02$), but no significant difference between the live and inactivated group (Table 8.1). Therefore, any behavioral alterations seen in mice given live and inactivated tachyzoites are not due to differences in body weight during testing, nor differences in antibody levels.

Table 8.1 Mouse weight and anti-*Toxoplasma* IgG serum levels

* denotes $p < 0.02$ as compared with infection controls.

	DPBS (n=10)	Live (n=9)	Adjuvant (n=7)	Inactivated (n=16)
Weight (g) 0 dpi	20.5 ± 0.5	19.7 ± 0.3	20.4 ± 0.5	19.3 ± 0.3
Weight (g) 60 dpi	28.1 ± 0.8	27.2 ± 0.7	29.3 ± 0.5	29.1 ± 0.5
Weight (g) 128 dpi	31.1. ± 0.8	29.8 ± 0.9	29.7 ± 0.7	29.4 ± 0.5
Anti-<i>Toxoplasma</i> IgG (O.D.)	0.02 ± 0.001	0.57 ± 0.067*	0.02 ± 0.001	0.34 ± 0.028*

8.4 Discussion

The present study indicates that *T. gondii* cysts do not have to persist in the brain for mice to exhibit behavioral changes. Apparently, the host immune response to *T. gondii* infection, in the absence of cyst formation, is a major contributor to behavior modifications. This thought is consistent with what has been seen in a study focused on decreased aversion to cat odor in *T. gondii* infected mice (22). However, it is still unclear whether some behaviors, such as anxiety, are due to the presence of *T. gondii* cysts in the brain or the immune system. This dilemma may be due to the methodology employed in our present study.

For instance, it is conceivable that the inactivated *T. gondii* tachyzoites may lead to the stimulation of some, but not all, toll-like receptors that are stimulated by the live parasites. Different toll-like receptors (TLRs) are stimulated by specific *T. gondii* proteins (e.g profilin stimulates TLR11 while glycosylphosphatidylinositols stimulate TLR2 and 4) (237, 238). In turn, each stimulated TLR triggers the secretion of specific cytokines. It is possible that while some proteins on the live and inactivated tachyzoites are both recognized by the host immune system, other proteins are recognized while the live parasite replicates and undergoes conversion to parasite cysts. Therefore, the live parasites may stimulate more TLRs and subsequently lead to the secretion of different cytokines than with the inactivated parasites.

Another possible explanation for the differences in anxiety between live and inactivated mice lies in the time in which the cytokines are up-regulated. For instance, the number of

tachyzoites administered to mice has been shown to affect what cytokines are up-regulated in serum by 6 hours post-infection (237). In our model, the numbers of live and inactivated tachyzoites first administered vary by 10^3 parasites. It is possible that this may result in an up-regulation of cytokines in the inactivated mice first, followed by cytokine up-regulation in the live infected mice days later when the parasite burden is high enough to elicit an immune response.

While there are caveats to our study, it is still clear that *T. gondii* does not have to be present in the brain as parasite cysts in order to have an impact on mouse behavior. It would be interesting to discern what specific *T. gondii* proteins are seen by the host, and in turn what TLRs are stimulated that lead to behavior modifications. A better understanding of how the host immune response to *T. gondii* infection affects behavior may open the way for a new line of therapies to be used in humans affected by *T. gondii*.

CHAPTER 9:

Concluding Remarks and Recommendations

Infection with the parasite *Toxoplasma gondii* (*T. gondii*) has been implicated in the pathogenesis of psychiatric disorders such as schizophrenia. While approximately 30-50% of the world's human population is infected with the parasite, not everyone infected develops schizophrenia and not everyone with schizophrenia has been infected. Human studies have been unable to explain how infection with *T. gondii* leads to mental illness in only some individuals. As rodents can be infected with *T. gondii* in a manner similar to humans, and can also be manipulated in a controlled environment, we sought to use mice to determine infection conditions that would lead to behavioral alterations, as a model of symptoms of psychiatric disorders. Much work has been done on using mice under various conditions to model aspects of schizophrenia in humans (Chapter 2). Some studies have also looked at the specific role of *T. gondii* infection on the development of cognitive deficits seen in patients with schizophrenia, although much of the work with mice has not been reproduced (Chapter 3). Irreproducibility in behavioral tests with *T. gondii* infected mice may be due to a lack of consistency in the parasite strain, mouse strain, age of infection, and behavioral testing conditions used between laboratories.

In the previous chapters of this dissertation I have shown that the strain of *Toxoplasma gondii* (*T. gondii*) (Chapter 4), the sex of the mouse (Chapter 5), the age in which the mouse is infected (Chapter 6), and the presence of *T. gondii* parasite cysts in the brain (Chapter 8) alter different mouse behaviors. While these findings provide insight into

infection conditions that lead to symptoms of schizophrenia, more work still needs to and should be done to refine the model.

As described in previous chapters, many of the behavioral alterations observed in *T. gondii* infected mice are subtle. This is exemplified by the need for psychostimulant administration to observe deficits in pre-pulse inhibition of the acoustic startle (Chapters 6 and 8). Subtle behavioral changes may be difficult to replicate within and between laboratories, and may reflect only slight changes in the neurobiology of the brain. Rather, a robust phenotype would suggest striking differences in the brain that would be easier to identify. In addition, it would provide a stronger basis for testing pre-clinical compounds and looking at mechanisms by which *T. gondii* infection will lead to deficits.

One avenue to pursue in creating a mouse model with a robust phenotype is infecting *T. gondii* into transgenic mice expressing genes associated with psychiatric disorders. Time and again studies have come out showing an environmental or genetic factor alone is insufficient to produce a behavioral phenotype in mice. However, when combined, the two have a synergistic effect and a phenotype can be observed (239). This may be true with *T. gondii* as well and should be tried.

In addition, more effort needs to go towards understanding the molecular underpinnings of behavioral effects of *T. gondii* infected rodents that are currently observed. While there are studies on the behavioral manipulation of *T. gondii*, and there is new work trying to understand how *T. gondii* infection alters the brain (240), there is no study combining the

two and clearly showing that specific brain alterations due to *T. gondii* infection are what is leading to behavioral changes. The field seems to be moving in this direction, and there is still much to be done.

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10.1016/j.bbi.2007.09.012. PubMed PMID: 18023140.

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10.1093/schbul/sbm008. PubMed PMID: 17322557; PubMed Central PMCID: PMC2526127.

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10.1016/j.chom.2008.01.001. PubMed PMID: 18312842.

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239. Kannan G, Sawa A, Pletnikov MV. Mouse models of gene-environment interactions in schizophrenia. *Neurobiol Dis*. 2013;57:5-11. Epub 2013/06/12. doi: S0969-9961(13)00154-X [pii] 10.1016/j.nbd.2013.05.012. PubMed PMID: 23748077; PubMed Central PMCID: PMC3737071.
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Curriculum Vitae

Johns Hopkins University School of Medicine

Geetha Kannan

Current as of June 2014

EDUCATION:

Johns Hopkins Medical Institute; Baltimore, MD
PhD candidate in Cellular and Molecular Medicine

August 2008- June 2014

Kenyon College; Gambier, OH
Bachelor of Arts in Molecular Biology
Cum Laude with Departmental High Honors

August 2003- May 2007

HONORS:

2011 International Behavioral Neuroscience Society Pre-doctoral Travel Award

2007 Sigma Xi Scientific Research Society

2007 Biology Award for Outstanding Service to the Department

Cum Laude

2005 Kenyon College Summer Science Scholar

2004 Omeris Student Poster Competition in Columbus, OH

2004 Summer Undergraduate Research Fellowship (SURF), at the University of Cincinnati

2004 SURF Outstanding Poster Award

2004 Leadership Award for the Red Cross Club

2004 Spring, 2004 Fall, 2006 Fall, 2007 Spring Kenyon College Merit List

PROFESSIONAL SERVICES:

Ad Hoc Journal Reviewer:

Parasitology Research

Folia Parasitologica

WORK EXPERIENCES:

Research:

Johns Hopkins Medical Institute; Baltimore, MD

August 2009-Present

Pre-doctoral research fellow under Dr. Mikhail Pletnikov

Division of Neurobiology and Biological Sciences

The aim of my work is to elucidate the role of *Toxoplasma gondii* infection on the development of psychiatric disorders through the use of mouse models. From this work I

have experience in mouse husbandry and genotyping, mouse behavior testing and analysis, organ and regional brain dissections, brain fixation and sectioning, immune and DAB staining, microscopy, *T. gondii* culturing, and western blotting. I am also in charge of mentoring undergraduate and high school students that join the laboratory.

National Institute of Mental Health (NIMH); Bethesda, MD July 2007- July 2008
Post-Baccalaureate Intramural Research Training Award fellow under Dr. Scott Young
Section on Neural Gene Expression

The aim of my work was to aid in creating a vasopressin receptor conditional knockout mouse. To this end I performed RNA extraction, Northern blot, Qrt-PCR, genotyping PCR, restriction digests, gene cloning, and transformations.

Kenyon College Department of Biology; Gambier, OH August 2003-May 2007
Research Assistant under Dr. Joan Slonczewski
Department of Biology, Microbiology Laboratory

The focus of my work was to characterize gene expression profiles of aerobically grown *E. coli* due to a temporal acid shift in internal pH. For this project I implemented and performed a method of RNA extraction new to the laboratory, microarray preparation and hybridization, advanced statistical analysis for microarrays, real-time PCR, bacterial transductions, survival assays, and fluorescent internal pH measurements.

University of Cincinnati Children's Hospital Research Foundation; 3333 Burnet Avenue; Cincinnati, OH June 2004 - August 2004
Summer Undergraduate Research Fellow under Dr. Masato Nakafuku
Department of Developmental Neurobiology

My project involved elucidating target genes of c-Myc in neurospheres. To accomplish this I designed primers for candidate genes, and performed PCR, reverse transcriptase PCR, mRNA extraction from cultured neurosphere cells.

Northern Kentucky University; Highland Heights, KY June 2003-July 2003
Summer Research Assistant under Dr. Mark Bardgett
Department of Psychology

The aim of the project was to determine whether exercise can contribute to neurogenesis in the hippocampus. My role was to BrDU mouse brain sections from a previous behavioral study, and count BrDU positive neurons via light microscopy.

Service and Teaching:

Johns Hopkins University; Baltimore, MD June 2012- August 2012
Immediate Supervisor for undergraduates in the Psychiatry Summer Training and Research (P-STAR) Program

Mentored one undergraduate in primer design, DNA extraction, PCR, gel electrophoresis, journal club presentation, and data presentation.

Johns Hopkins University; Baltimore, MD June 2012- August 2012
Immediate Supervisor for undergraduates in the Stanley Summer Scholars Program
Mentored one undergraduate in conducting behavioral tests on mice and data presentation.

Johns Hopkins University; Baltimore, MD June 2011- August 2011
Immediate Laboratory Supervisor in the Stanley Summer Scholars Program
Mentored two undergraduates in sectioning mouse brains, immunofluorescent staining, and data presentation.

Johns Hopkins University; Baltimore, MD June 2010-August 2010
Immediate Laboratory Supervisor in the Incentive Mentoring Program Summer Program
Mentored an inner city Baltimore high school student in genotyping PCR and data presentation.

Johns Hopkins University; Baltimore, MD June 2009-August 2009
Immediate Laboratory Supervisor
Mentored a high school student in genotyping PCR, cell culture, and data presentation.

CentroNia; Washington, DC August 2007- May 2008
Middle school math and reading tutor

Northern Kentucky University; Highland Heights, KY June 2006
Teaching Assistant for English Language Learners Fun with Science Camp
Department of Biological Sciences
Assisted with laboratory preparation and setup, errands, and instructing the high school students on laboratory molecular techniques.

Kenyon College Department of Biology; Gambier, OH January 2006- May 2006
Introductory Biology Laboratory Teaching Assistant
Department of Biology
Assisted with laboratory set up and instructed students on safe laboratory technique, paper writing, data analysis, and power point.

PUBLICATIONS:

Kannan G, Prandovszky E, Gressit KL, Yolken RH, Severance EG, Jones-Brando L, Pletnikov MV. One minute ultraviolet exposure inhibits *Toxoplasma gondii* tachyzoite replication and cyst conversion without diminishing host immune response. *Experimental Parasitology*. *Accepted pending revisions*.

Kannan G, Sawa A, Pletnikov MV. Mouse models of gene-environment interactions in schizophrenia. *Neurobiol Dis*. 2013.

Severance EG, **Kannan G**, Gressitt KL, Dickerson FB, Pletnikov MV, Yolken RH. Antibodies to food antigens: Translational research in psychiatric disorders. *Neurology, Psychiatry and Brain Research*. 2012; 18(2):87-88.

Severance EG, **Kannan G**, Gressitt KL, Xiao J, Alaedini A, Pletnikov MV, et al. Anti-gluten immune response following *Toxoplasma gondii* infection in mice. *PLoS One*. 2012;7(11):e50991. PMCID: 3510169.

Xiao J, Viscidi RP, **Kannan G**, Pletnikov MV, Li Y, Severance EG, et al. The *Toxoplasma* MAG1 peptides induce sex-based humoral immune response in mice and distinguish active from chronic human infection. *Microbes Infect*. 2012;15(1):74-83.

Kannan G, Pletnikov MV. *Toxoplasma gondii* and cognitive deficits in schizophrenia: an animal model perspective. *Schizophr Bull*. 2012;38(6):1155-61. PMCID: 3494063.

Xiao J, **Kannan G**, Jones-Brando L, Brannock C, Krasnova IN, Cadet JL, et al. Sex-specific changes in gene expression and behavior induced by chronic *Toxoplasma* infection in mice. *Neuroscience*. 2012;206:39-48.

Pogorelov VM, Nomura J, Kim J, **Kannan G**, Ayhan Y, Yang C, et al. Mutant DISC1 affects methamphetamine-induced sensitization and conditioned place preference: a comorbidity model. *Neuropharmacology*. 2011;62(3):1242-51. PMCID: 3115479.

Abazyan B, Nomura J, **Kannan G**, Ishizuka K, Tamashiro KL, Nucifora F, et al. Prenatal interaction of mutant DISC1 and immune activation produces adult psychopathology. *Biol Psychiatry*. 2010;68(12):1172-81. PMCID: 3026608.

Kannan G, Moldovan K, Xiao JC, Yolken RH, Jones-Brando L, Pletnikov MV. *Toxoplasma gondii* strain-dependent effects on mouse behavior. *Folia Parasitol (Praha)*. 2010;57(2):151-5.

Kannan G, Wilks JC, Fitzgerald DM, Jones BD, Bondurant SS, Slonczewski JL. Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. *BMC Microbiol*. 2008; 8:37. BMCID: 2270276.

PRESENTATIONS:

Oral Presentations:

Kannan, Geetha. *Toxoplasma* or the Immune Response, which leads to changes in mouse behavior? Implications for drug development. Oral presentation at the 19th annual Stanley Medical Research Institute Meeting in Baltimore, MD. December 2013.

Kannan, Geetha. Toxoplasma Mouse Models of Schizophrenia. Short talk at the Johns Hopkins University Schizophrenia Center Seminar Series in Baltimore, MD. April 2013.

Kannan, Geetha. Animal Models of Toxoplasma Infection. Oral presentation at the 18th annual Stanley Medical Research Institute Meeting in Baltimore, MD. December 2012.

Kannan, Geetha. Toxoplasma gondii Mouse Model of Schizophrenia-like Neurobehavioral Abnormalities in Mice: Gender-Related Effects and Molecular Correlates. Oral presentation at the International Behavioral Neuroscience Society in Steamboat Springs, CO. May 2011.

Kannan, Geetha. Crossing Kingdoms: A plant virus that can infect mammals? Oral presentation at the Stanley Medical Research Institute Meeting in Ann Arbor, MI. March 2011.

Kannan, Geetha. Toxoplasma gondii infection on male and female mouse behavior. Oral presentation at the Stanley Medical Research Institute Meeting in Ann Arbor, MI. March 2011.

Kannan, Geetha. Toxoplasma gondii infection in mice: Parasite-strain and mouse gender related behavioral alterations. Oral presentation at the Johns Hopkins University Cellular and Molecular Medicine Program lunch series in Baltimore, MD. January 2011.

Kannan, Geetha. *Toxoplasma gondii* infection in mice: Parasite-strain and mouse-gender related behavioral alterations. Oral presentation at the 16th annual Stanley Meeting in Chevy Chase, MD. December 2010.

Kannan, Geetha. *Toxoplasma gondii* mouse model of schizophrenia: Strain dependent effects. Oral presentation at the Johns Hopkins University Psychiatry Potpourri in Baltimore, MD. May 2010.

Poster Presentations:

Kannan, Geetha, Ye Li, J-C Xiao, Emily G. Severance, Lorraine Jones-Brando, Robert H. Yolken, and Mikhail V. Pletnikov. Schizophrenia-like abnormalities in mice due to adolescent exposure to *Toxoplasma gondii*. Poster presented at the International Behavioral Neuroscience Society in Dublin, Ireland. June 2013.

Kannan, Geetha, Kristin L. Gressitt, Emese Prandovszky, Robert H. Yolken, Emily G. Severance, Lorraine Jones-Brando, and Mikhail V. Pletnikov. UV inactivation of *Toxoplasma gondii* tachyzoites: method for studying the contribution of the host immune response to behavioral changes in mice. Poster presented at the 12th International Congress on Toxoplasmosis in Oxford, England. June 2013.

Yang, Chun-Xia, Jun Nomura, Bagrat Abazyan, **Geetha Kannan**, Akira Sawa, Mollie Meffert, Mikhail V. Pletnikov. DISC1 modulates innate immune response in the brain. Poster presented at the 41st Society for Neuroscience in Washington, DC. November 2011.

Kannan, Geetha, J-C Xiao, Irina N. Krasnova, Emily Severance, Jean-Lud Cadet, Robert Yolken, Lorraine Jones-Brando, Mikhail V. Pletnikov. Schizophrenia-like neurobehavioral abnormalities in mice due to *Toxoplasma gondii* infection. Poster presented at the 41st Society for Neuroscience in Washington, DC. November 2011.

Kannan, Geetha, J-C Xiao, Irina N. Krasnova, Jean-Lud Cadet, Robert Yolken, Lorraine Jones-Brando, Mikhail V. Pletnikov. *Toxoplasma gondii* mouse model of schizophrenia-like neurobehavioral abnormalities in mice: gender-related effects and molecular correlates. Poster presentation at the International Behavioral Neuroscience Society in Steamboat Springs, CO. May 2011.

Kannan, Geetha., Priyanka Shah, J-C Xiao, Sarven Sabuncuyan, Irina Krasnova, Jean Cadet, Robert Yolken, Lorraine Jones-Brando, and Mikhail V. Pletnikov. *Toxoplasma gondii* mouse model of schizophrenia. Poster presented at the 40th Society for Neuroscience in San Diego, CA. November 2010.

Kannan, Geetha, Jessica C. Wilks, Sandra S. BonDurant, Brian D. Jones, and Joan L. Slonczewski. Acid-Shift Time Course of Gene Expression Profiles of Aerobically Grown *Escherichia coli*. Poster Presented at the American Society for Microbiology 106th General Meeting in Orlando, FL. May 2006.

Kannan, Geetha, Motoshi Nagao, and Masato Nakafuku. Search for Downstream Targets for c-Myc in Neural Stem Cells. Poster presented at the BioOhio 2004 Entrepreneurs and Innovation Student Poster Competition sponsored by OMERIS in Columbus, OH. October 2004

Kannan, Geetha, Motoshi Nagao, and Masato Nakafuku. Search for Downstream Targets for c-Myc in Neural Stem Cells. Poster presented at The Summer Undergraduate Research Program at the University of Cincinnati College of Medicine. Awarded Outstanding Poster Award. August 2004.

Other Posters:

Nomura, Jun, Martin Ma, Sofya Abazyan, Bagrat Abazyan, **Geetha Kannan**, Chun-Xia Yang, Akira Sawa, Solomon Snyder, Mikhail V. Pletnikov. Towards DISC1 functions in astrocytes. Poster presented at the 41st Society for Neuroscience in Washington, DC. November 2011.

Nomura, Jun, Bagrat Abazyan, **Geetha Kannan**, K. Ishizuka, Kelly Tamashiro, Fred Nucifora, Vladimir Pogorelov, Irina Krasnova, Jean Cadet, Carlos Pardo-Villamizar, S. Mori, A. Kamiya, M. Vogel, Christopher Ross, Mikhail V. Pletnikov. Prenatal interaction of mutant DISC1 and immune activation produces adult psychopathology. Poster presented at the 40th Society for Neuroscience in San Diego, CA. November 2010.

Abazyan, Bagrat, Jun Nomura, **Geetha Kannan**, Chunxia Yang, Akira Sawa, Christopher A. Ross, Mikhail V. Pletnikov. A mouse model of astrocyte dysfunction in psychiatric disorders. Poster presented at the 40th Society for Neuroscience in San Diego, CA. November 2010.

Romano, Julia D., Bao Lige, **Geetha Kannan**, Isabelle Coppens. Modifications of host cell functions by intravacuolar Toxoplasma. Poster presented at the 10th International Congress on Toxoplasmosis in Kerkrade, The Netherlands. June 2009.

Lee, Heon-Jin, **Geetha Kannan**, Scott Young, M. Palkovits. Differential gene expression profiling in Avpr1b knockout mouse brain. Poster presented at the Society for Neuroscience Conference in Washington, DC. November 2008.

Young, Scott, Heon-Jin Lee, **Geetha Kannan**, S.J. Lolait, A. Iacangelo, Emily Shepard, A.-M. O'Carroll. Triple knockouts of the mouse vasopressin 1a, vasopressin 1b and oxytocin receptors are fertile and useful reagents for antibody controls. Poster presented at the Society for Neuroscience Conference in Washington, DC. November 2008.

Kannan, Geetha, Jessica C. Wilks, Sandra S. BonDurant, Brian D. Jones, and Joan L. Slonczewski. Acid-Shift Time Course of Gene Expression Profiles of Aerobically Grown *Escherichia coli*. Poster Presented at the Gordon Conference in New London, NH. July 2006.

Muzny, Ashley, Michael Riddle, Janet Gowdy, **Geetha Kannan**, and Mark Bardgett. Effects of Exercise on Neurogenesis in Mice with Hippocampal Damage. Poster Presented at the Celebration of Student Research and Creativity, Northern Kentucky University, April 2004.

LEADERSHIP ROLES:

Incentive Mentoring Program (IMP)

Spring 2011- Spring 2012

Director of College Preparation and Career Planning

I led a team of approximately ten individuals in providing resources for IMP mentors to prepare their inner city Baltimore high school mentees for college.

South Asian Multicultural Organization for Student Awareness Fall 2005-Spring 2007
Co-President

In charge of organizing a large all campus events (Diwali) and smaller events requiring communication with other club members as well as businesses we were to employ, and filling out paperwork.

Biology Journal club Fall 2005-Spring 2007
Co-President

In charge of communicating with faculty, student presenters, and other club members, publicity, and ordering food.